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ABSTRACT

The menstrual cycle phase of breast cancer resection affects the frequency of cancer metastatic spread. Tumor metastases are 2-3 fold more frequent when the resection is performed during diestrus as compared to estrus. Tumor angiogenesis is essential for both cancer growth and lethal metastatic cancer spread. The balance between vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) modulates new blood vessel formation and blood vessel permeability. In a nonbreast tumor, methylcholantrenene(meth). The changes in breast cancer capillary permeability, VEGF and bFGF that occur during each fertility cycle, in breast tissue and breast cancer, putatively in response to cyclical changes in sex hormones, may contribute, at least in part, to both the modulation of cancer growth and post-resection breast cancer spread by the fertility cycle. Scope: We hypothesize that there are characteristic patterns of tumor cell gene expression that change throughout the menstrual (estrous) cycle, and that a subset of these cycling genes are in part associated with, and responsible for the changes in curability of resected breast cancers. We investigated the influence of the estrous cycle on breast tumor surgical cure and metastatic spread to the lungs, using a primary, transplantable, mammary carcinoma, resected for surgical cure from young, sexually mature cycling C3HeB/FeJ female mice at each of 4 fertility cycle stages. Therefore, we asked whether VEGF and/or bFGF concentrations and levels of gene expression in mammary tumors and normal mammary tissue are modulated rhythmically by the mouse fertility cycle in ways that might help explain the dependence of post-resection breast cancer spread upon the murine estrous and, by analogy, human menstrual cycle. Major Findings: A 96% surgical cure frequency was documented when the tumor is resected during estrus The second best surgical cure rate is achieved when tumors are resected during metestrus (79% overall cure rate). From these findings, we conclude that the optimal timing of surgical resection resides within that span at and following ovulation associated with maximum fertility and the highest pulsatile levels of LH, FSH, prolactin and subsequent high albeit falling levels of estrogen and progesterone. This luteal span is about 1-1.5 days in the mouse and 7-14 days long in the woman. We have validated that VEGFA and bFGF are modulated, at the message and/or protein levels, within breast cancer cells by the estrous cycle, in ways that may help explain the fact that breast cancer resection during the luteal phase of the reproductive cycle is 25% more frequently cured that if it is resected during the follicular phase when VEGFA angiogenesis is most robust.

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Introduction

In the cycling C3H mouse and the menstrual cycle of the premenopausal woman, the timing of breast cancer resection within the estrous cycle of determines to some extent whether the disease is cured in the mouse and the ten-year disease free survival of the woman. Two metaanalyses have demonstrated the positive effect of timing breast cancer resection as near to midcycle (early luteal phase) as possible. A third study has estimated this beneficial effect of surgery timing to be potentially more than two-fold greater than the positive effect of adjuvant chemotherapy. Recent editorial review demonstrates that established prognostic indicators including: tumor histopathologic grade, tumor cell proliferation markers, tumor cell estrogen and progesterone receptor content, tumor cell molecular characteristics associated with angiogenesis and tumor cell invasion and motility are more frequently ominous and more severely negative in tumors resected in the follicular as compared to the luteal phase of the patient's menstrual cycle.

Breast cancer growth and spread is affected by the mammalian fertility cycle (1-3). Tumor growth is consistently slower during estrus than during diestrus in a transplantable mouse breast cancer model (1) . In this model, the timing of resection of equal sized breast cancers, within the estrous cycle, determines the frequency with which the cancer metastasizes following resection. Two to three times as many mice are cured by primary tumor resection performed at or near estrus, as compared to when cancers are resected at diestrus.(3, 4) Clinical data indicate that the timing of breast cancer surgery during the menstrual cycle meaningfully affects breast cancer control(5-11). In aggregate, the most high quality retrospective clinical studies, two meta-analyses and the single prospective study done to date, demonstrate an average absolute 25% ten year disease free survival advantage for premenopausal women whose breast cancers are resected during early luteal phase of their menstrual cycle, as compared to the follicular phase(12-14).

It is not yet known how the estrous and menstrual cycles modulate cancer growth and post-resection metastatic potential. We do know, however, that tumor blood vessel permeability and angiogenesis are each essential for cancer growth and spread(15). We also know that progesterone(P_4) estrogen(E_2) modulate new blood vessel formation and capillary permeability in the uterus and ovary(16). These sex hormones may, therefore, regulate the growth and post-resection spread of breast cancer cells, at least in part, by stimulating the production of angiogenesis modulating molecules such as vascular endothelial growth factor (VEGF) and/or basic fibroblast growth factor (bFGF) within breast cancers. There is reason to believe that the balance between or ratio of these two molecules may be largely responsible for microvasculature changes essential for both tumor growth and metastasis following primary cancer resection(16).

Poor breast cancer outcomes, therefore, is independently predicted by high VEGF tumor levels and low bFGF levels. The relationship between the concentrations of these two potentially sex hormone regulated angiogenesis modulators with, in some circumstances, opposite action may, help to explain the fertility cycle stage dependence of breast cancer growth and post-resection spread. Therefore, we asked whether VEGF and/or bFGF concentrations and levels of gene expression in mammary tumors and normal mammary tissue are modulated rhythmically by the mouse fertility cycle in ways that might help explain the dependence of post-resection breast cancer spread upon the murine estrous and, by analogy, human menstrual cycle.

Body

Breast Cancer Outcome Curability and the Cycle Stage of Primary Tumor Resection, Results in Women. We discovered a relationship between circadian timing and/or cycle stage of resection in both the mouse(*17-19*) and in the woman.(*5-10*) We have since demonstrated many of the potential mechanisms of this cyclical biology. These include: 1) the modulation of

cellular immune function by the cycle, including natural killer cell function and interleukin-2 production;(*19*) 2) Breast cancer growth rate;(*20*) and 3) Breast cancer capillary permeability and the molecular regulation of new blood vessel formation.(*20*)

The Reproductive Cycle Affects the Host-Surgery-Cancer Interaction (Our Model). When a transplantable mammary carcinoma of C_3HeB/FeJ mice is resected after several weeks of growth, not every mouse is cured and some die subsequently from metastases, not unlike the human situation.(*21*) The timing within the fertility cycle of surgical resection of the breast tumor influences whether subsequent metastases occur.(*17*) An estrogen- and progesterone receptor-positive mammary tumor cell line derived from this primary tumor also demonstrates this same biology.(*22*) Estrous cycle coordination of host factors, like new blood vessel formation, may be, relevant to the cycle stage dependence of metastatic cancer spread.(*23*)

Breast Cancer Growth Rate Cycles. We have studied a transplantable, spontaneous breast cancer of C_3HeB/FeJ mice (3 studies), and a transplantable methylcholantherene induced sarcoma of CD2F1 mice (2 studies). We concurrently measured local cancer size and estrous cycle stage up to twice and at least once each day. There is a natural individual variability in the average length of normal estrus (3-1/2 to 7 days) cycle in mice. We assessed the effect of the cycle stage and cycle duration on tumor size. We have found identical estrous cycle stage coordination of cancer size, and identical effects of cycling frequency across all studies in each of these two tumors, both of which express estrogen receptor alpha and progesterone receptor. Little or no change in cancer size occurs during proestrus (pre-ovulatory phase) and estrus (peri-ovulatory phase); tumor size increases several fold during diestrus (post-ovulatory phase); and the tumor shrinks partially as the next proestrus phase approaches. Across both mouse strains and tumor types, mice whose average cycle length is shorter (faster cyclers), have slower average tumor growth rate than slower cyclers (Table 1, Figure 1).(*20*)

The virtually identical modulation of tumor size and cancer growth rate are recognized in each of two very different transplantable cancers (one, classically sex-hormone-dependent and potentially metastatic breast tumor; and the other, never previously recognized as hormone dependent and locally aggressive sarcoma) growing in two unrelated inbred mouse strains, indicates that the fertility cycle related host factors affect cancer size and growth rate. The fertility cycle influence upon tumor biology and the host-cancer balance is apparently not limited to tumors of breast or tissues of endocrine origin, and is thereby, a phenomenon of more general significance. Although we have documented the presence of estrogen and progesterone receptors in both of these tumors, whether this cyclical behavior exists in ER negative and/or PR negative tumors remains to be determined. Faster cyclers demonstrate two-fold slower tumor growth rate than slower cyclers. These data are consistent with epidemiologic findings associating menstrual cycle length and breast cancer risk; faster cycling is associated with the lower subsequent breast cancer risk.(*24, 25*) In summary, both fertility cycle stage and cycling frequency affect the growth rates of two different experimental cancers. If these findings, which are consistent with early clinical observation, are also clinically relevant, then, the effect of the menstrual cycle on cancer growth and post-resection cancer spread may be a general one, not limited to breast cancer.

Previous Studies Documenting Post Surgical Cure/Metastatic Potential Cycles as Function of Cycle Stage Timing. We investigated the influence of the estrous cycle on breast tumor surgical cure and metastatic spread to the lungs, using a primary, transplantable, mammary carcinoma, resected for surgical cure from young, sexually mature cycling C_3HeB/FeJ female mice at each of 4 fertility cycle stages. Oophorectomized (Ovx) animals were also used to determine the effect of minimal E_2 and P_4 levels on metastatic potential.

In these two studies, a 96% surgical cure frequency was documented when the tumor is resected during estrus (Figure 2). The second best surgical cure rate is achieved when tumors are resected during metestrus (79% overall cure rate).(*17*) These results further suggest a probable role for circulating E_2 and P_4 levels in modulating the metastatic process. From these

findings, we conclude that the optimal timing of surgical resection resides within that span at and following ovulation associated with maximum fertility and the highest pulsatile levels of LH, FSH, prolactin and subsequent high *albeit* falling levels of estrogen and progesterone. This luteal span is about 1-1.5 days in the mouse and 7-14 days long in the woman.

Molecular Mechanisms Linking Angiogenesis, Cancer Growth and Spread to the Cycle. It is not known how the estrous and menstrual cycles modulate cancer growth and post-resection metastatic potential. We do know, however, that tumor blood vessel permeability and angiogenesis are each essential for cancer growth and spread.(*15, 26, 27*) We also know that progesterone(P_4) estrogen(E_2) modulate new blood vessel formation and capillary permeability in the uterus and ovary.(*16*) These sex hormones may, therefore, regulate the growth and post-resection spread of breast cancer cells, at least in part, by stimulating the production of angiogenesis modulating molecules such as vascular endothelial growth factor (VEGFA) and/or basic fibroblast growth factor (bFGF) within breast cancers.(*16*)

VEGFA is a mitogen specific for vascular endothelial cells, and a known enhancer of vascular permeability.(*28*) VEGFA mRNA and protein levels are regulated by E_2 and P_4, in rat uterus.(*29, 30*) VEGFA is produced by human and rodent breast cancer cells(*31*). Increased tumor cell VEGFA expression and increased microvessel density in primary breast cancer are each associated with decreased patient survival.(*31-33*) In mice, shutting down VEGFA effects, either with antisense VEGFA oligonucleotides or monoclonal antibody to VEGFA, decreases tumor blood vessel density and tumor growth rate and diminishes the frequency of metastasis.(*34, 35*) Conversely, over-expression of VEGFA enriches tumor vasculature, growth and increases tumor vessel permeability and enhances metastatic cancer spread.(*36*) Tumor vascular permeability is reduced within hours by VEGFA antibody, strongly suggesting that maintenance of tumor vessel integrity requires the presence of VEGFA within the tumor microenvironment.(*37*) Successfully metastatic tumor cells must traverse these vessels at least twice during metastatic transposition. Therefore, the VEGFA modulation of vascular integrity may be necessary for cancer spread.

bFGF is both a mitogen for a variety of cell types, including the endothelial cell, and, in many circumstances, a negative modulator of angiogenesis.(*38*) bFGF mRNA and protein levels are regulated by E_2 and P_4, in rat uterus.(*29*) bFGF is present in human mammary tumor cytosol(*39*). High tumor bFGF levels are associated with high breast cancer estrogen receptor (ER) concentrations, low grade(good prognosis) histopathology, and small primary tumors(*39*). Patients whose tumors contain high concentrations of bFGF protein show better breast cancer survival. This is also true when patients' tumors demonstrate high bFGF mRNA levels.(*40, 41*) A low bFGF level in breast carcinoma is an independent indicator of poor prognosis, adumbrating early disease recurrence and death.

***VEGFA Expression in Tumors (C_3H Mammary Tumor and Meth A Sarcoma). VEGFA Protein Levels*.** Overall, mammary tumor VEGFA protein levels were > 100 fold higher than normal mammary tissue levels in C_3H mice (12.9-24.7 vs 0.03-0.09 pg/mg). VEGFA protein levels in mammary tumor samples from proestrus mice (estrogen and progesterone rich) were nearly 2-fold higher (24.7 ± 3.1) compared to tumors obtained from the estrogen-poorer stages of metestrus (14.9 ± 1.4) and diestrus (12.9 ± 1.8, $F=6.5$, $p=0.001$; Table 2 and Figure 3). Oophorectomy (ovx) did not further diminish tumor VEGFA levels compared to tumors obtained during the lowest estrogen and progesterone phase, diestrus in C_3H mice($p=0.12$, Table 2). We also analyzed VEGFA protein as a function of the fertility cycle in a totally different tumor model, meth A sarcoma in CD_2F_1 female mice. A similar and significant fertility cycle difference in tumor VEGFA protein ($F=3.42$, $p=0.024$) was seen in this sarcoma tumor by immuno-histochemical analysis, quantitated by image analysis (Figure 4a). Sarcoma tumors showed higher VEGFA protein levels in proestrus and diestrus not unlike the pattern seen in the mammary tumors (Figures 5b, c). Both of these tumors, by standard immuno-histochemical analysis, stain positive for estrogen receptor alpha, negative for estrogen receptor beta and

positive for progesterone receptor (data not shown) and show estrous cycle dependence of tumor growth rates.(*20*)

VEGFA mRNA levels. Mammary tumor VEGFA mRNA levels did not vary prominently within the fertility cycle (F=1.3, p=0.28; Table 2). VEGFA mRNA levels in tumors from ovx animals were not significantly different from tumors obtained during diestrus (p=0.56; Table 2).

VEGFA, bFGF protein and RNA relationships between tissues across the estrous cycle. VEGFA is a secreted protein; therefore, in tumor bearing mice, VEGFA concentrations are five times higher in serum than in breast cancer cells, and they are several hundred times higher in cancer cells than in normal mouse breast cells. The concentration of this molecule is modulated by the estrous cycle most prominently in cancer, where it peaks in proestrus. It, however, falls precipitously to much lower values in estrus, when surgical curability is surest. Unlike in breast cancer, VEGFA peaks in estrous in normal breast cells. Basic FGF concentrations in normal breast are six times higher than in breast cancer. The concentration of this protein is most prominently modulated by the estrous cycle in normal breast in which it peaks during metestrus. The VEGFA and bFGF RNA both cycle in normal breast tissue. The bFGF RNA in breast cancer cells cycles prominently, while the VEGFA RNA expression changes less robustly in these cancer cells throughout the estrous cycle.

Mammary Tumor Vessel Density, Blood Content and Capillary Permeability. To determine if the variations observed in tumor VEGFA protein levels over the fertility cycle were associated with differences in tumor vascularity, we examined mammary tumor CD31 positive blood vessel density, blood volume and capillary permeability in tumors obtained across the estrous cycle. There was not significant differences in the density of tumor blood vessels across the cycle (Table 2). The pattern of mammary tumor blood volume was higher in estrus (0.069±0.013 ml blood/g tissue) and lowest in proestrus (0.043±0.015 ml blood/g tissue), but these differences did not, however, reach statistical significance. Mammary tumor capillary permeability, varies with the cycle and is some 50% higher in diestrus, the cycle phase associated with the highest frequency of post-resection metastasis (F=3.9, p =0.01; Table 2).

Circulating Sex Hormones. Estrogen and progesterone, expressed as percentage of the mean, are double plotted along with surgical cure rates (frequency) at each estrous cycle phase in Figure 5. Estrogen and progesterone concentrations changes occurring throughout the mouse estrous cycle have been well documented. We did not measure these hormones in our mice, however, we have retrieved published values.(*42, 43*) The mean values and standard errors for estrogen are: proestrous:10.3±1.1, estrous: 4.2±1.0, metestrus: 3.1±0.7 and diestrus:7.5±1.3 pg/ml.(*43*) Progesterone at onset of activity are extrapolated from data published by Michael, S, 1976(*42*), with levels during proestrus of 60, estrus of 8, metestrus of 20, and diestrus of 8(ng/ml). Both estrogen and progesterone are highest during proestrus and fall between proestrus and estrous, remain low during metestrus, then estrogen rises while progesterone remains low in diestrus (Figure 5).

Tumor Growth Rate. Growth rates were calculated from serially assessed mammary tumor sizes of C_3H mice as a function of biologic time (estrous cycle stage and cycle number) of measurement from our previous study.(*20*) Standardized tumor growth rates vary with fertility cycle phase (Table 2, Figure 7). This cyclic effect is consistent across three separate studies of the C_3H mammary tumor. Average tumor growth rates across many estrous cycles are significantly different($p < 0.001$) and are 2-3 fold higher in diestrus (636.2 $\pm$ 54 mm3/day), as compared to other cycle stages (proestrus 214.8 $\pm$ 30, estrus 308.4 $\pm$ 38, metestrus 276.1 $\pm$ 33 mm3/day, Table 2). Estrous stage tumor growth across all cycles showed that tumor growth rate is highest in diestrus, that cycle phase associated with low surgical curability (Figure 7). A similar dependence of tumor size/growth rate on fertility cycle is seen in the meth A sarcoma(Figure 1).(*20*)

Estrous Cycle Pattern of Post-Resection Breast Cancer Spread. Two large, independent studies to determine the optimal timing of breast cancer resection within the fertility cycle have recently been published.(*17*) In these studies, 33% (6/18) of mice resected during proestrus remained free of metastases and apparently cured, 96% (25/26) of mice resected during estrus were apparently cured, 79% (11/14) of mice resected in metestrus remained metastasis free, and 44% (11/25) of those resected in diestrus were apparently cured (Table 2, Figure 7). These surgical cure proportions are significantly different across the four estrous stages (χ^2=24.6, p<0.001). Ovx also impacted cure with a cure frequency of 50% (5/10) (χ^2=24.9, p<0.001).(*17*)

The Relationship of Ratios of Sex Hormones (E_2/P_4) and Angiogenesis Modulators (bFGF/VEGFA) to Capillary Permeability, Tumor Growth and Post-Resection Cancer Spread Throughout the Estrus Cycle. The ratio of serum estrogen to progesterone, tumor capillary permeability, and tumor growth rate covary throughout the cycle almost perfectly. Figure 7 shows the highest serum estrogen/progesterone ratio, the highest tumor capillary permeability and the fastest tumor growth rate each occur in tumors during diestrus. Diestrus is that time within the cycle when post-resection spread is most likely. Estrus, the phase of the cycle when 96% of the mice are cured by resection, is associated with lower serum estrogen/progesterone ratios, lower tumor capillary permeability and lower tumor growth rates.

During each cycle, the ratio of tumor bFGF/VEGFA protein also changes. The highest ratios occur during estrus and metestrus when tumor spread after resection is least likely. This ratio in the tumor falls during diestrus when surgical cure is least frequent and tumor growth rate and tumor capillary permeability are each highest.(*44*)

The fertility cycle alters mammary tumor growth and post-resection spread in both mice and human beings.(*2, 17, 20*) Others have shown how hormone concentrations vary throughout the estrous cycle.(*42, 43*) During each estrous cycle, the concentrations of estrogen and progesterone peak concurrently in proestrus in response to pulsatile hypothalamic secretion of FSH, LH and other hormones. Estrogen and progesterone remain high but fall rapidly during estrus, after follicular rupture, when the ova are available for fertilization. These highly compressed murine cycle phases are roughly comparable to the luteal phase of the menstrual cycle. If fertilization does not occur, a new crop of estrogen secreting follicles are built and estrogen rises in the absence of progesterone during metestrus and falls again in diestrus.

Adjacent estrous cycle phases of highest cure frequency (estrus, metestrus) are preceded by the highest levels and most rapid declines of both estrogen and progesterone. The cycle phases associated with most frequent post-surgical cancer spread are those phases of lowest progesterone levels, and low and rising unopposed estrogen.

Those cycle phases associated with high and/or rapidly rising VEGFA are those associated with the highest risk of post-resection breast cancer spread, leaky tumor capillaries and fastest cancer growth rate (diestrus, proestrus). We further show that these fertility cycle effects on tumor VEGFA protein are not limited to mammary tumors, since we find similar cycle dependent differences in a sarcoma.

Discontinuous, intermittent, in fact, saltatory, growth seems to be characteristic of how biologic systems organize growth. Traverse through the cell cycle is a necessarily saltatory process at the microscopic level; where as saltation and stasis characterize human growth, at the organismic level.(*45*) Our data demonstrate that relevant negative and positive molecular regulators of angiogenesis and tumor capillary permeability, cancer growth rate, and post-resection metastatic cancer spread are each modulated by the mammalian fertility cycle. These covariations do not prove causation. Conclusions about causation await specific manipulation of estrogen, progesterone, and modulation tumor cell VEGFA. If that specific VEGFA blockade eliminates or the fertility cycle dependence of cancer growth and spread and at the same time diminishes the post-resection breast cancer cure frequency, causation will be likely and might lead to the testing of peri-resection anti-angiogenic therapies to improve breast cancer cure frequency.

Statement of Work

Tumor microdissection and RNA Preparation

1. microdisect frozen tumors resected from experimental animas at different phases of the estrous cycle.
2. prepare RNA from tumors using dynel poly-A beads
3. prepare double-stranded cDNA
4. check quality of cDNA's from various tumors using Real-Time Quantitative PCR

High Quality cDNA

5. Prepare SAGE libraries from two to four cDNA samples selected from the above set
6. Send SAGE libraries outside sequencing facility for data collection SAGE libraries in the mail
7. Analyze gene expression data using bioinformatics tools to determine statistically significantly differentially expressed genes

KEY RESEARCH ACCOMPLISHMENTS:

- WE have validated that VEGFA and bFGF are modulated, at the message and/or protein levels, within breast cancer cells by the estrous cycle, in ways that may help explain the fact that breast cancer resection during the luteal phase of the reproductive cycle is 25% more frequently cured than if it is resected during the follicular phase when VEGFA angiogenesis is most robust.

REPORTABLE OUTCOMES

1. **Graduate Studies** of Peter Miller (MS), Elizabeth Green (MS), Young Oh (on going) project
2. **Published Articles and Conference Presentation**
   - Wood PA, **Hrushesky WJM**. Sex cycle modulates cancer growth. *Breast Can Res Treat* 2005; 91(1):95-102.
   - Wood PA, Bove K, You S, Chambers A, **Hrushesky WJM**. Cancer growth and spread are saltatory and phase-locked to the reproductive cycle through mediators of angiogenesis. *Mol Cancer Ther* 2005; 4(7):1065-75.
   - Restsky MW, Demicheli R, **Hrushesky WJM**. Does surgery induces angiogenesis in breast cancer? Indirect evidence from bimodal relapse pattern for untreated early breast cancer patients and the mammography paradox for women age 40-49. *Int J Surgery* 2005; 3(3):179-87.
   - Baum M, Demicheli R, **Hrushesky W**, Retsky M. Does Surgery Unfavorably Perturb the "Natural History" of Early Breast Cancer by Accelerating the Appearance of Distant Metastases? *European J Cancer* 2005; 41:508-515.

CONCLUSIONS

- We have validated changes in the molecular modulators, VEGFA and bFGF, that serve as cells at both the message and protein levels.
- Our data demonstrate that relevant negative and positive molecular regulators of angiogenesis and tumor capillary permeability, cancer growth rate, and post-resection metastatic cancer spread are each modulated by the mammalian fertility cycle.
- These results have led to an application to the VA MERIT program to determine estrogen and progesterone can modulate breast cancer cell VEGFA expression in vitro and in vivo and whether this modulation can lead to the enhanced surgical curability of breast cancer.

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42. S. D. Michael, *Proc Soc Exp Biol Med* **153**, 254 (1976).
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44. P. A. Wood, K. Bove, S. You, A. Chambers, W. J. Hrushesky, *Mol Cancer Ther* **4**, 1065 (Jul, 2005).
45. M. Lampl, J. D. Veldhuis, M. L. Johnson, *Science* **258**, 801 (1992).

APPENDICES.

The following articles are attached:

- Wood PA, **Hrushesky WJM**. Sex cycle modulates cancer growth. *Breast Can Res Treat* 2005; 91(1):95-102.
- Wood PA, Bove K, You S, Chambers A, **Hrushesky WJM**. Cancer growth and spread are saltatory and phase-locked to the reproductive cycle through mediators of angiogenesis. *Mol Cancer Ther* 2005; 4(7):1065-75.
- Baum M, Demicheli R, **Hrushesky W**, Retsky M. Does Surgery Unfavorably Perturb the "Natural History" of Early Breast Cancer by Accelerating the Appearance of Distant Metastases? *European J Cancer* 2005; 41:508-515.
- Restsky MW, Demicheli R, **Hrushesky WJM**. Does surgery induces angiogenesis in breast cancer? Indirect evidence from bimodal relapse pattern for untreated early breast cancer patients and the mammography paradox for women age 40-49. *Int J Surgery* 2005; 3(3):179-87.

SUPPORTING DATA

Tables

Table 1. Effect of Fertility Cycle Stage and Cycling Frequency upon Tumor Size and Growth Rate.

Tumor Model	Fertility Cycle Stage						Cycling Freq Tumor Growth Rate (*F, p*)
	Tumor Size (% Mean of Each Cycle)						
	Proestrus	Estrus	Metestrus	Diestrus	ROC	*ANOVA (F, p)*	
C3H Breast Tumor							
Study 1 (n=40)	46.9 ± 7.5	37.8 ± 7.1	73.2 ± 7.9	219.5 ± 29.7	5.8	24.2, <0.001	5.4, 0.001
Study2 (n=120)	23.4 ± 2.4	38.2 ± 3.6	72.8 ± 6.3	207.1 ± 13.6	8.9	86.3, <0.001	5.3, <0.001
5 cycles (n=8)	88.9 ± 25.1	79.6 ± 31.0	99.6 ± 47.3	137.1 ± 30.5	0.0	NS	
4 cycles (n=33)	69.3 ± 15.0	73.0 ± 14.3	71.0 ± 13.8	167.8 ± 21.2	2.4	8.8, <0.001	
3 cycles (n=44)	56.1 ± 9.2	73.3 ± 11.6	69.5 ± 9.8	170.5 ± 15.7	3.0	18.8, <0.001	
2 cycles (n=33)	43.5 ± 9.7	50.1 ± 9.6	70.8 ± 10.2	193.0 ± 18.8	4.4	27.3, <0.001	
1 cycles (n=9)	4.4 ± 3.4	16.3 ± 6.7	71.5 ± 17.3	219.4 ± 32.6	50.0	18.3, <0.001	
	CD2F1 Meth A Sarcoma						
Study 1 (n=16)	50.2 ± 7.1	61.4 ± 14.9	105.2 ± 15.4	184.6 ± 33.6	3.7	9.4, <0.001	3.2, 0.043
Study 2(n=86)	58.6 ± 4.2	76.1 ± 6.4	121.4 ± 17.1	149.1 ± 26.2	2.5	6.9, <0.001	4.9, 0.001
Uterus	**Uterine Weight (% Mean of Cycle)**						
	C3H tumor bearing mice						
Study 2(n=120)	150.1± 21.5	168.4 ± 5.7	118.6 ± 5.7	88.0 ± 2.4	1.9	41.6, <0.001	
Study 3(n=100)	125.3± 11.0	107.0 ± 4.9	88.1 ± 4.6	104.1 ± 7.2	1.4	4.4, 0.006	

Table 2: Cancer biology, sex hormone concentrations, angiogenic growth factors, blood vessel density and capillary permeability in breast cancer and normal C3H mammary tissue (plus VEGFA in meth A sarcoma).

	Proestrus	Estrus	Metestrus	Diestrus	Analysis results (across 4 estrous cycles) X^2 value	p value	Ovx	T-test (di vs. ovx) p value
Cancer Biology								
Surgical Cure (%)[a]	33 (n=18)	96(n=26)	79(n=14)	44(n=10)	24.6	<0.001	50(n=10)	nd
					F value	**p value**		
Cancer growth rate (mm3/day)[b]	214.8±30.1	308.4 ± 38.4	276.1 ±33.5	636.2± 54.3	21.1	<0.001	nd	nd
Hormones	**means ± SE**						**mean ± SE**	
Circulating Estrogen(pg/ml)[c]	10.3±1.1	4.2±1.0	3.1±0.70	7.5±1.3	nd	nd	nd	nd
Circulating Progesterone(ng/ml)[d]	60	8	20	8	nd	nd	nd	nd
Growth Factors								
a. VEGF								
tumor protein (pg/mg)	24.7 ± 3.1	16.1 ± 1.2	14.9 ± 1.4	12.9 ± 1.8	6.5	0.001	12.6 ± 1.0	0.12
tumor mRNA (PI units)[e]	0.53 ± 0.13	0.70 ± 0.1	0.81 ± 0.06	0.75 ± 0.1	1.3	0.28	0.67 ± 0.09	0.56
normal mammary protein (pg/mg)	0.03 ± .002	0.09 ± 0.03	0.05 ± .007	0.05 ± .008	2.4	0.08	0.03 ± .002	0.01
normal mammary mRNA (PI units)	0.35 ± .07	0.32 ± .03	0.17 ± .02	0.21 ± .04	3.3	0.03	0.25 ± .06	0.48
serum protein (pg/ml)	158 ± 17.3	201 ± 51.1	134 ± 28.8	244 ± 74.6	0.98	0.42	98.9 ± 17.3	0.09
b. meth A Sarcoma VEGF								
tumor protein optical density[f]	0.054 ± 0.005	0.033 ± 0.006	0.043± 0.008	0.056± 0.005	3.42	0.024	nd	nd
c. bFGF								
tumor protein (pg/mg)	0.29 ± 0.05	0.44± 0.06	0.52 ± 0.07	0.36± 0.09	1.2	0.34	0.24 ± .06	0.07
tumor mRNA (PI units)	0.51 ± 0.11	0.33 ± 0.1	0.33 ± 0.05	0.2 ± .03	4.4	0.01	0.17 ± .02	0.46
normal mammary protein (pg/mg)	1.9 ± 0.11	2.1 ± 0.42	3.3 ± 0.58	2.8 ± 0.32	2.6	0.06	1.7 ± 0.08	0.01
normal mammary mRNA (PI units)	0.19 ± .04	0.25 ± .03	0.14 ± .02	0.13 ± .02	4.1	0.01	0.10 ± .02	0.67
Tumor CD31 blood vessel	8.2 ± 0.65	6.21 ± 0.75	7.51 ± 0.71	8.1 ± 0.38	1.85	0.15	7.3 ± 1.38	0.54
Tumor Vascular volume[g]	0.043 ± .015	0.069 ± .013	0.049 ± .007	0.063 ± .009	1.1	0.35	nd	nd
Tumor capillary permeability[h]	0.11 ± .02	0.14 ± .01	0.12 ± .01	0.16 + .01	3.9	0.01	nd	nd

[a]Bove. BCRT 2002,75:65-72; [b]Wood. BCRT 2005,91(1):95-102; [c]Bergman. Endocrinology 1992, 130:1923-30; [d]Michael. Proc Soc Exp Med 1976, 153:254-7;
[e]PI Units = relative phosphoimage units. Tissue specific PCR samples are processd simultaneously for a specific gene so that comparisons may be made across estrous cycle and expressed relative to control gene for each sample; [f]VEGF meth A sarcoma (IHC), density over a high power field (all other data are from C_3H tissue/tumor); [g](ml Blood/g tissue); hextravasuclar plasma volume/g tissue/hr; Ovx-oophorectomized mice; nd- not done.

Figures

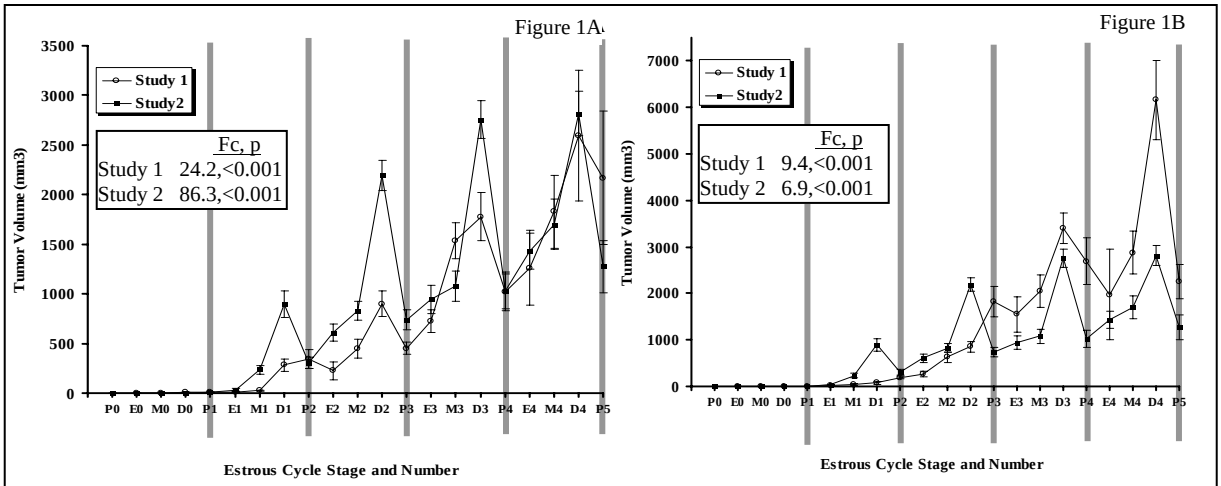


Figure 1. The effect of estrous cycle stage upon tumor size. Subcutaneous mammary tumor volumes in C_3HeB/FeJ female mice (**A**) and meth A sarcoma tumor volumes in CD_2F_1 female mice (**B**) vary as a function of fertility cycle stage. (Source: Wood PA. BCRT 2005;91:95-102).

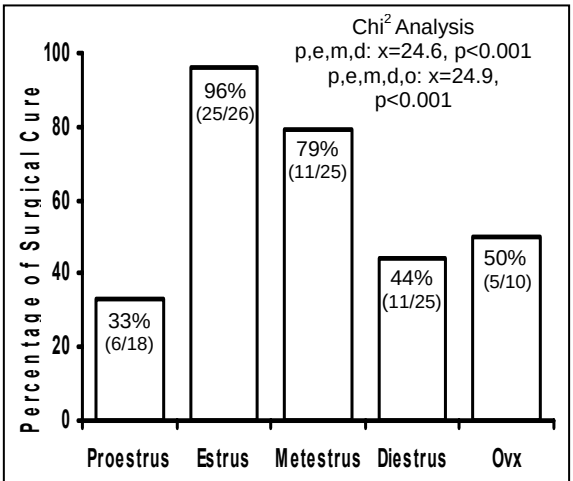


Figure 1. The effect of estrous cycle stage at tumor resection on surgical cure. (Source: Bove. BCRT 2005;75:65-72.)

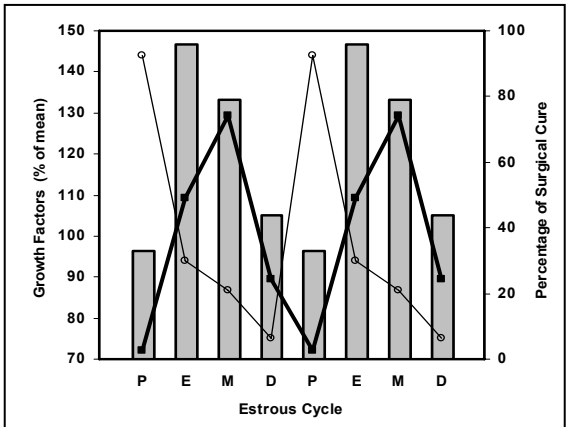
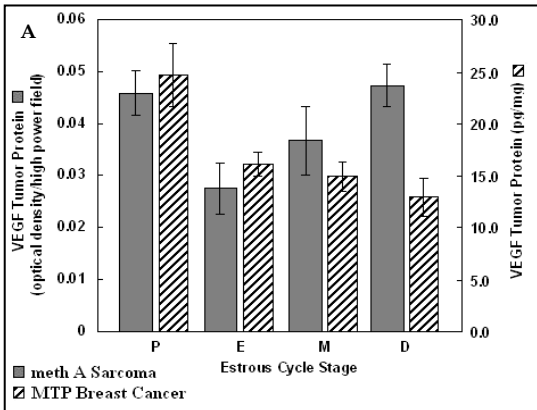


Figure 2. Double plot of the relationship between C_3H mammary tumor VEGFA (—○—) and bFGF (—■—) protein concentration (as % of mean values) plotted on top of surgical curability(▬)of this tumor across the estrous cycle.



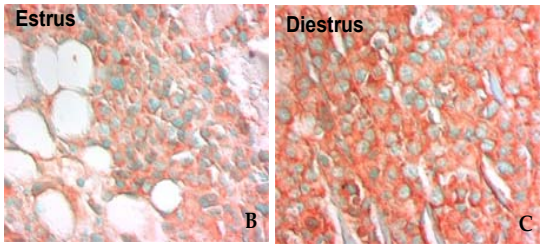


Figure 3. VEGFA protein derived by immuno-histochemical staining in meth A sarcoma tumors and enzyme immunoassay in tumor homogenates for MTP mammary tumors.

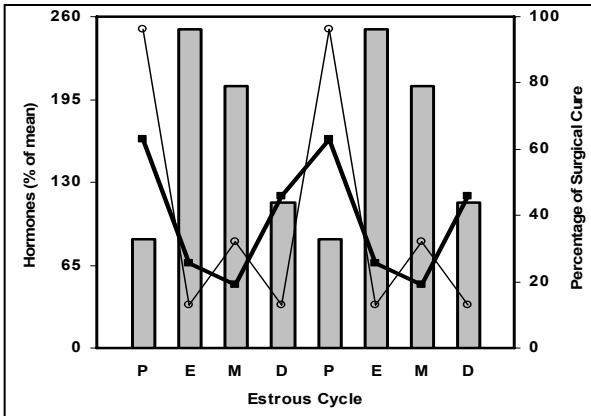


Figure 4. Double plot of the relationship between serum concentrations of estrogen(E_2, —■—) and progesterone(P_4, —○—) in the mouse across the estrous cycle and % surgical curability(▭) of C_3H mice with local, resected MTP mammary tumors (Table 2).

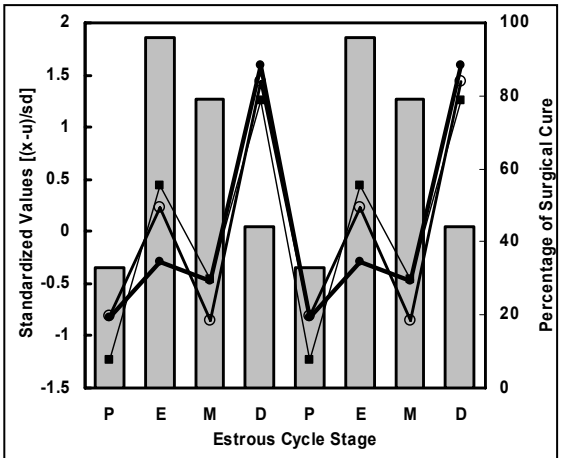


Figure 5. Co-variation of the serum estrogen/progesterone (—○—) ratio; capillary permeability (—■—) of tumor vasculature; and, the rate of tumor growth(—◆—) and surgical curability(▭) at each of the four estrous cycle phases.

Cancer growth and spread are saltatory and phase-locked to the reproductive cycle through mediators of angiogenesis

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Abstract

The frequency of breast cancer metastatic spread is affected by the menstrual cycle phase of its resection. Breast cancer growth, post-resection spread, and cure frequency are each modulated by the estrous cycle in C_3HeB/FeJ mice. Tumor metastases are 2- to 3-fold more frequent when the resection is done during diestrus as compared with estrus. Tumor angiogenesis is essential for both cancer growth and lethal metastatic cancer spread. The balance between vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF) modulates new blood vessel formation and blood vessel permeability. Sex hormones modulate the expression of these key angiogenesis regulators in the endometrium and uterus. We, therefore, asked whether the estrous cycle modulates the density of CD31-positive vessels within the tumor, the permeability of tumor blood vessels, levels of VEGF and bFGF immunoreactive protein in normal breast and breast cancer, and whether expression of these genes are modulated by the estrous cycle stage in C_3HeB/FeJ mice. We find that tumor blood vessel density and blood volume do not vary throughout the cycle; however, tumor capillary permeability is regulated by the estrous cycle being highest in diestrus, the cycle stage associated with the highest cancer growth rate and the highest frequency of post-resection cancer metastasis. VEGF protein levels in breast cancer are >100-fold higher than in normal breast. VEGF protein in this mammary tumor varies with the estrus cycle with highest levels in proestrus. In a non-breast tumor, methylcholantrenene A sarcoma, from CD_2F_1 mice, tumor VEGF protein also varies with the estrus cycle with highest levels in proestrus and diestrus. VEGF gene expression in the mammary tumor does not change significantly across the cycle, but is modulated by the cycle in normal breast tissue. bFGF protein concentration is 6-fold higher in normal breast than in breast cancer. bFGF protein pattern in both tumor and breast are similar, opposite to VEGF, and affected by oophorectomy. bFGF message is modulated by the cycle in both breast cancer and normal breast. The changes in breast cancer capillary permeability, VEGF, and bFGF that occur during each fertility cycle, in breast tissue and breast cancer, putatively in response to cyclical changes in sex hormones, might contribute, at least in part, to both the modulation of cancer growth and post-resection breast cancer spread by the fertility cycle. These fertility cycle–induced effects on tumor biology also seem to extend to non–breast cancer biology. [Mol Cancer Ther 2005;4(7):1065–75]

Introduction

The mammalian fertility cycle affects breast cancer growth and spread (1–3). In a transplantable mouse breast cancer model, tumor growth is consistently slower during estrus than during diestrus (1). In this model, the timing of resection of equal sized breast cancers, within the estrous cycle, determines the frequency with which the cancer metastasizes following resection. Two to three times as many mice are cured by primary tumor resection done at or near estrus, as compared with when cancers are resected at diestrus (3, 4). Clinical data indicate that the timing of breast cancer surgery during the menstrual cycle meaningfully affects breast cancer control (5–11). In aggregate, the most high-quality retrospective clinical studies, two metaanalyses and the single prospective study done to date, show an average absolute 25% 10-year disease-free survival advantage for premenopausal women whose breast cancers are resected during early luteal phase of their menstrual cycle, as compared with the follicular phase (12–14).

We do not know how the estrous and menstrual cycles modulate cancer growth and post-resection metastatic potential. We do know, however, that tumor blood vessel permeability and angiogenesis are each essential for cancer growth and spread (15). We also know that progesterone and estrogen modulate new blood

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vessel formation and capillary permeability in the uterus and ovary (16). These sex hormones might, therefore, regulate the growth and post-resection spread of breast cancer cells, at least in part, by stimulating the production of angiogenesis modulating molecules such as vascular endothelial growth factor (VEGF) and/or basic fibroblast growth factor (bFGF) within breast cancers. There is reason to believe that the balance or the ratio between these two molecules may be largely responsible for microvasculature changes essential for both tumor growth and metastasis following primary cancer resection (16).

VEGF is a mitogen specific for vascular endothelial cells, and a known enhancer of vascular permeability (17). VEGF mRNA and protein levels are regulated by estrogen and progesterone, in rat uterus (18, 19). VEGF is produced by human and rodent breast cancer cells (20). Increased tumor cell VEGF expression and increased microvessel density in primary breast cancer are each associated with decreased patient survival (20, 21). In mice, shutting down VEGF effects, either with antisense VEGF oligonucleotides or monoclonal antibody to VEGF, decreases tumor blood vessel density and tumor growth rate and diminishes the frequency of metastasis (22, 23). Conversely, overexpression of VEGF enriches tumor vasculature, growth, increases tumor vessel permeability, and enhances metastatic cancer spread (24). Tumor vascular permeability is reduced within hours by VEGF antibody, strongly suggesting that maintenance of tumor vessel integrity requires the presence of VEGF within the tumor microenvironment (25). Successfully metastatic tumor cells must traverse these vessels at least twice during metastatic transposition. Therefore, the VEGF modulation of vascular integrity may be necessary for cancer spread.

bFGF is both a mitogen for a variety of cell types, including the endothelial cell, and, in many circumstances, a negative modulator of angiogenesis (26). bFGF mRNA and protein levels are regulated by estrogen and progesterone, in rat uterus (18). bFGF is present in human mammary tumor cytosol (27). High tumor bFGF levels are associated with high breast cancer estrogen receptor concentrations, low grade (good prognosis) histopathology, and small primary tumors (27). Patients whose tumors contain high concentrations of bFGF protein show better breast cancer survival. This is also true when patients' tumors show high bFGF mRNA levels (28, 29). A low bFGF level in breast carcinoma is an independent indicator of poor prognosis, adumbrating early disease recurrence and death.

Therefore, high VEGF tumor levels and low bFGF levels independently predict poor breast cancer outcomes. The relationship between the concentrations of these two potentially sex hormone–regulated angiogenesis modulators with, in some circumstances, opposite action may, help to explain the fertility cycle stage dependence of breast cancer growth and post-resection spread. Therefore, we asked whether VEGF and/or bFGF concentrations and levels of gene expression in mammary tumors and normal mammary tissue are modulated rhythmically by the mouse fertility cycle in ways that might help explain the dependence of post-resection breast cancer spread upon the murine estrous and, by analogy, the human menstrual cycle.

Materials and Methods

C_3H Mammary Tumor Model

Sexually mature, female C_3HeB/FeJ mice (The Jackson Laboratory, Bar Harbor, ME), 10 to 14 weeks of age, were housed four per cage alongside singly housed male mice, to enhance estrous cycling as in our previous studies (3, 4). All procedures were done in the same quadrant (14 hours after lights on) of the circadian cycle (time of day) because of the known variation of the immune response, surgical response, and tumor behavior with circadian time (30). All animals were kept on lighting schedules with 12 hours light alternating with 12 hours of dark with food and water freely available. In a subgroup of mice (see Table 1, row 1), bilateral oophorectomy was done (n = 10) at 10 weeks of age. Confirmation of oophorectomy was accomplished through serial vaginal cytology, described below. The primary mammary tumor (B. Fisher, University of Pittsburgh, Pittsburgh, PA) originated spontaneously in a female C_3H mouse and has subsequently been passed *in vivo* in C_3HeB/FeJ female mice (31). Tumors were harvested under sterile conditions and tumor cell suspensions made by gentle grinding of minced tumor pieces over a stainless steel mesh into Medium 199 (Life Technologies, Gaithersburg, MD). Tumor cells were inoculated s.c. at 2×10^4 viable cells in the right hind flank. Tumor sites were palpated and subsequently measured twice daily at 12-hour intervals (length, width, and height) by the same individual, using calipers, and estrous cycle stage was concurrently determined by vaginal smear. Average tumor growth rates were then computed for each estrus cycle stage. Tumors were excised from animals in one of four estrous stages (n = 12/stage) and ovariectomized animals (n = 15/stage), at an average size of 1,300 mm^3. Serum was recovered and stored at −80°C. Lower mammary gland from the opposite side of s.c. tumor was isolated from C_3H tumor–bearing animals. Histologic examination confirmed the tissue as normal mammary gland devoid of any tumor. All resections were done without any knowledge of the estrous cycle phase at the time of that resection (blinded).

CD_2F_1 Sarcoma Tumor Model

Female CD_2F_1 mice 10 to 14 weeks old were purchased from Charles River (Portage, MI). The animals were housed four per cage and maintained in a 12-hour light, 12-hour dark cycle with food and water ad libitum. Ascitic tumor cell suspension of methylcholantrenene A sarcoma was harvested from BALB/c female mouse, centrifuged and resuspended in DMEM and 5×10^5 cells were inoculated s.c. on the backs of mice during the activity cycle. Animals were sacrificed at mean tumor size of 990 mm^3 at one of

Table 1. Cancer biology, sex hormone concentrations, angiogenetic growth factors, blood vessel density, and capillary permeability in breast cancer and normal C_3H mammary tissue (plus VEGF in methylcholantrenene A sarcoma)

	Proestrus	Estrus	Metestrus	Diestrus	Analysis results (across four estrous cycles)		Oophorectomized mice	*t* test (diestrus versus oophorectomy)
					F value	*P*		*P*
Cancer biology								
Surgical cure (%)*	33 (n = 18)	96 (n = 26)	79 (n = 14)	44 (n = 10)	24.6†	<0.001	50 (n = 10)	nd
Cancer growth rate (mm^3/d)‡	214.8 ± 30.1	308.4 ± 38.4	276.1 ± 33.5	636.2 ± 54.3	21.1	<0.001	nd	nd
	Means ± SE						Means ± SE	
Hormones								
Circulating estrogen (pg/mL)§	10.3 ± 1.1	4.2 ± 1.0	3.1 ± 0.70	7.5 ± 1.3	nd	nd	nd	nd
Circulating progesterone (ng/mL)‖	60	8	20	8	nd	nd	nd	nd
Growth factors								
VEGF								
Tumor protein (pg/mg)	24.7 ± 3.1	16.1 ± 1.2	14.9 ± 1.4	12.9 ± 1.8	6.5	0.001	12.6 ± 1.0	0.12
Tumor mRNA (PI units)¶	0.53 ± 0.13	0.70 ± 0.1	0.81 ± 0.06	0.75 ± 0.1	1.3	0.28	0.67 ± 0.09	0.56
Normal mammary protein (pg/mg)	0.03 ± 0.002	0.09 ± 0.03	0.05 ± 0.007	0.05 ± 0.008	2.4	0.08	0.03 ± 0.002	0.01
Normal mammary mRNA (PI units)	0.35 ± 0.07	0.32 ± 0.03	0.17 ± 0.02	0.21 ± 0.04	3.3	0.03	0.25 ± 0.06	0.48
Serum protein (pg/mL)	1.58 ± 17.3	201 ± 51.1	134 ± 28.8	244 ± 74.6	0.98	0.42	98.9 ± 17.3	0.09
Methylcholantrenene A sarcoma VEGF								
Tumor protein absorbance**	0.054 ± 0.005	0.033 ± 0.006	0.043 ± 0.008	0.056 ± 0.005	3.42	0.024	nd	nd
bFGF								
Tumor protein (pg/mg)	0.29 ± 0.05	0.44 ± 0.06	0.52 ± 0.07	0.36 ± 0.09	1.2	0.34	0.24 ± 0.06	0.07
Tumor mRNA (PI units)	0.51 ± 0.11	0.33 ± 0.1	0.33 ± 0.05	0.2 ± 0.03	4.4	0.01	0.17 ± 0.02	0.46
Normal mammary protein (pg/mg)	1.9 ± 0.11	2.1 ± 0.42	3.3 ± 0.58	2.8 ± 0.32	2.6	0.06	1.7 ± 0.08	0.01
Normal mammary mRNA (PI units)	0.19 ± 0.04	0.25 ± 0.03	0.14 ± 0.02	0.13 ± 0.02	4.1	0.01	0.10 ± 0.02	0.67
Tumor CD31 blood vessel	8.2 ± 0.65	6.21 ± 0.75	7.51 ± 0.71	8.1 ± 0.38	1.85	0.15	7.3 ± 1.38	0.54
Tumor vascular volume ††	0.043 ± 0.015	0.069 ± 0.013	0.049 ± 0.007	0.063 ± 0.009	1.1	0.35	nd	nd
Tumor capillary permeability ‡‡	0.11 ± 0.02	0.14 ± 0.01	0.12 ± 0.01	0.16 + 0.01	3.9	0.01	nd	nd

Abbreviation: nd, not done.
*Ref. 3.
†χ^2 value.
‡Ref. 1.
§Ref. 41.
‖Ref. 42.
¶PI units, relative phosphoimage units. Tissue-specific PCR samples are processed simultaneously for a specific gene so that comparisons could be made across estrous cycle and expressed relative to control gene for each sample.
**VEGF methylcholantrenene A sarcoma (immunohistochemistry), density over a high power field (all other data in this table are from C_3H tissue/tumor).
†† Milliliters of blood per gram of tissue.Extravascular plasma volume per gram of tissue per hour.
‡‡ Extravascular plasma volume per gram of tissue per hour.

four estrous stages (n = 10-20/stage). Tumors were dissected away from skin and underlying muscle and fixed in 10% buffered formalin for 24 hours and embedded in paraffin blocks.

Fertility Cycle Phase Determination

Daily vaginal smears were done using sterile saline washings stained with Diff Quik (Baker, Newark, DE), and were read by one individual. Slides from each mouse were read in sequence to determine the orderly progression of cycling and to classify each of those smears as either proestrus (P), estrus (E), metestrus (M), or diestrus (D; refs. 3, 32). Estrous stage was determined daily, starting 4 days prior to tumor inoculation until sacrifice to confirm regular cycling in each mouse and to assign the most precise estrous stage determination at the time of sacrifice. In our previous studies with these tumor models, estrous cycling continues regularly with minimal perturbation until just prior to death when a slight prolongation of the cycle length is observed only in the last week of life (33).

Reverse Transcription-PCR

Tissues were rapidly collected, homogenized and total RNA recovered (Trizol, Life Technologies). First-strand cDNA was generated from 1.0 μg of total RNA using SuperScript II reverse transcriptase (Life Technologies). Quantitative PCR was done using the GeneAmp DNA Amplification Reagent Kit (Perkin-Elmer, Norwalk, CT) with ^{32}P-labeled dCTP. Oligonucleotide paired primers for mouse VEGF, bFGF, ribosomal protein S16, and histone H1 were purchased from Life Technologies. PCR samples were fractionated by electrophoresis on an 8% PAGE and quantitated by phosphorimage analysis (STORM 860, Molecular Dynamics, Sunnyvale, CA). The linear range of amplification was determined for each tissue and each primer pair. Results are expressed as the ratio of the gene of interest to control gene for each sample (ribosomal S16 for tumor samples, and histone H1 for normal mammary tissue samples as levels of S16 varied significantly across the fertility cycle in normal mammary tissue but not in tumor samples).

VEGF/bFGF Immunoassay

Concentrations of VEGF in mouse serum and tissue homogenates were quantified using a ''QuantikineM'' mouse VEGF immunoassay (R&D Systems, Inc., Minneapolis, MN). Concentrations of mouse bFGF in tissue homogenates were quantified using a Quantikine human bFGF immunoassay (R&D Systems). Tissues were homogenized on ice in buffer containing 50 mmol/L Tris-HCl, 0.5% NP40, 1 mmol/L DTT, 100 mmol/L NaF, 0.1 mmol/L Na_3VO_4, and protease inhibitor cocktail (Mini-protean, Boehringer Mannheim (Indianapolis, IN)). The 12,000 × g supernatant was collected and assayed.

Tumor Tissue Array

A trained clinical and experimental pathologist examined H&E sections from each tumor and marked the most viable areas of the tumor tissue. These areas were aligned with the tumor specimen within each tissue block for tissue array core sampling. A tissue array instrument (Beecher Instruments, Inc., Sun Prairie, WI) was used to sample and transfer the paraffin-fixed tissue cores into predrilled holes on a recipient paraffin block. For each tumor block, a tissue core was taken, labeled by position, and arrayed side by side in the recipient block. Multiple 5-μm sections were cut from the array block and mounted on the positively charged glass slides (SurgiPath, Richmond, IL) for histopathologic and immunochemical examination.

VEGF Protein Immunohistochemistry

The histopathologically selected tissue array sections, after deparaffinization and hydration, were digested using pepsin (4 mg/mL in 0.01 N HCl solution) for 40 minutes and washed in PBS twice (pH 7.2) for 5 minutes. Endogenous peroxidase activity was blocked by 3% H_2O_2 in PBS for 15 minutes. Slides were incubated in normal goat serum for 1 hour at room temperature. The primary antibody (anti-VEGF mouse monoclonal antibody, Santa Cruz Biotechnology, Santa Cruz, CA), was applied to sections at 1:400 dilution and incubated overnight at 4°C. The secondary biotinylated rabbit anti-mouse antibody and ExtrAvidin-peroxidase (B-6398 and E-8386, Sigma, St. Louis, MO) were applied for 45 and 30 minutes at room temperature, respectively. Between incubations, slides were washed thrice (5 min/each) in PBS. The color was developed by AEC substrate (AEC-101 kit, Sigma). The sections were finally counterstained with 1% methyl green solution (Sigma). The same tissue array slides were stained without primary antibody as negative controls.

CD31 Immunohistochemistry

CD31 is expressed in new blood vessels. Tumors were collected, fixed in 10% buffered formalin for <24 hours and embedded in paraffin for immunohistochemical analysis (CD31 staining) as previously described (21, 34, 35). Blood vessels staining positively for CD31 were then morphometrically enumerated.

Quantitation of Immunostain

The immunostained tumor tissue array sections were viewed under the Axioskop microscope. A digital image was taken from each of tumor tissue core using AxioVision (Carl Zeiss, Germany) and analyzed using SigmaScan Pro4 (SPSS, Inc., Chicago, IL). The target objectives in this image were defined and selected by a preset intensity. The average intensity of the objectives ($Obj_{intensity}$) was measured. Images were also taken from the coordinated negative control stain sections to estimate background intensity ($B_{intensity}$). The final formula for calculating specific VEGF immunostain intensity was (36–38):

$$Obj'_{intensity} = (\log 255 - \log Obj_{intensity}) - (\log B_{intensity})$$

Tumor Blood Volume and Capillary Permeability Determination

^{59}Fe-labeling of homologous RBC was accomplished by i.p. injection of 0.5 μCi ^{59}Fe-chloride (New England Nuclear, NEN, Newton, MA) into non–tumor-bearing C_3HeB/FeJ blood donor mice followed 48 hours later by exsanguination under methoxyflurane anesthesia and euthanasia. The blood was washed twice with PBS and

^{59}Fe labeled RBC (~0.5 μCi) were mixed with an aliquot of ^{125}I-labeled bovine albumin (1-4 μCi, NEN) and given by tail vein injection to tumor-bearing mice 1 hour before euthanasia. Following exsanguination, the radioactivity of each isotope per volume of central blood and per gram wet weight of tissue was determined in the tumor using a gamma counter. Blood content and capillary leak was determined by dilution, as previously described (39, 40).

Tumor Growth Rate throughout the Estrus Cycle

We borrowed data on tumor size measurements and fertility cycle stage of C_3HeB/FeJ female mice in our previous study (1). In that study, MTP breast tumor cells were inoculated s.c. into the right flank and three-dimensional tumor size (TS = length × width × height) was measured by calipers from the time of tumor appearance until sacrifice. Measurements were made once daily at 14 hours after lights on. Vaginal smears were obtained from mice at the time of tumor inoculation and at each tumor measurement. Different estrous cycle stages, successive cycle numbers (cycle number 1, 2, 3, etc.) and estrous stages within each of these cycles (P1, E1, M1, D1; P2, E2, M2, D2; etc.) were assigned starting from the time of tumor inoculation until the last measurement. Daily tumor growth rate is then obtained as the increment in tumor size from the preceding tumor size measurement. Average tumor growth rate during each phase of the estrous cycle is then computed by considering all estrous cycle numbers.

Post-Resection Curability/Metastatic Potential throughout the Estrus Cycle

These results were taken from a series of published trials (3). Surgical cure frequency was expressed as the percentage of mice without evidence of cancer spread at autopsy, >30 days after the last death from metastatic breast cancer, verified by autopsy.

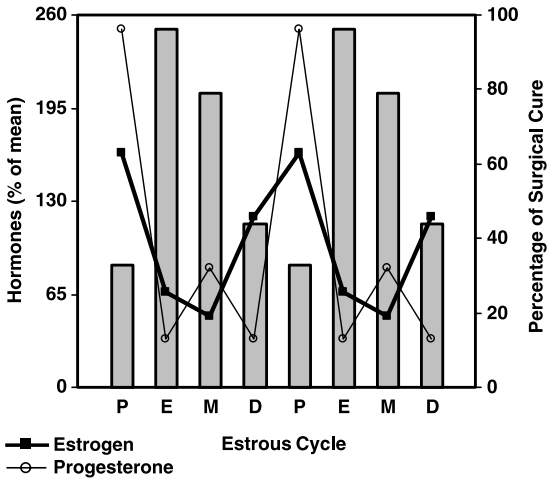

Figure 1. Double plot of the relationship between serum concentrations of estrogen and progesterone in the mouse across the estrous cycle (41, 42) and percentage surgical curability (*columns*) of C_3H mice with local and resected MTP mammary tumors (3). Numerical data in Table 1. Stages: *P,* proestrus; *E,* estrus; *M,* metestrus; *D,* diestrus.

Sex Hormone Concentrations during the Estrous Cycle

These values were obtained from two reports (41, 42). Frequent serum measurements of estrogen and progesterone were made in each of these papers by sacrificing groups of mice at frequent intervals (up to every 20 minutes). For our purposes, the values during each vaginal smear identifiable cycle phase were averaged and/or extrapolated. These averages appear in Table 1 and Fig. 1 presented as the percentage of overall estrous cycle mean value.

Statistical/Parametric Analyses

Numerical values were contrasted across the four estrous cycle phases using one way ANOVA with a commercially available statistical program (SuperANOVA). When two groups were compared with one another (i.e., diestrous stage obtained tissues versus tissues from ovariectomized animals), Student's two-tailed t test was done (SuperANOVA). A $P \leq 0.05$ is considered statistically significant. Patterns of hormones and growth factors are double-plotted along the estrous cycle. Double plotting of rhythmic patterns is a standard chronobiological technique that allows visualization of recurring patterns. In order to visually examine rhythmic covariations of tumor growth, hormone, and growth factor values were correspondingly standardized and plotted simultaneously. Standardized rates were obtained as $(x - u) / \mathrm{SD}$, where x is the estrous stage mean, u the overall mean across all estrous stages.

Results

Angiogenic Growth Factor Expression

VEGF Serum Levels in Tumor-Bearing C_3H Mice

Because a prior study in humans reported plasma VEGF protein levels to vary throughout the menstrual cycle in normal women (43), we wondered if serum VEGF levels would be affected by the estrous cycle. Serum VEGF protein levels did not vary significantly in C_3H mammary tumor bearing mice with fertility cycle stage or oophorectomy state, as determined by immunoassay (P = 0.42, Table 1). We could not obtain unclotted blood from these mice for practical reasons, and thereby have no data on plasma concentrations of this molecule. The amount of serum VEGF produced by blood clotting–associated platelet activation and aggregation is substantial, and could thereby mask estrous cycle differences.

VEGF Expression in Tumors (C_3H Mammary Tumor and Methylcholantrenene A Sarcoma)

VEGF Protein Levels. Overall, mammary tumor VEGF protein levels were >100-fold higher than normal mammary tissue levels in C_3H mice (12.9-24.7 versus 0.03-0.09 pg/mg). VEGF protein levels in mammary tumor samples from proestrus mice (estrogen- and progesterone-rich) were nearly 2-fold higher (24.7 ± 3.1) compared with tumors obtained from the estrogen-poorer stages of metestrus (14.9 ± 1.4) and diestrus (12.9 ± 1.8, F = 6.5, P = 0.001; Table 1; Fig. 2). Oophorectomy did not further diminish tumor VEGF levels compared with tumors obtained during the lowest estrogen and progesterone

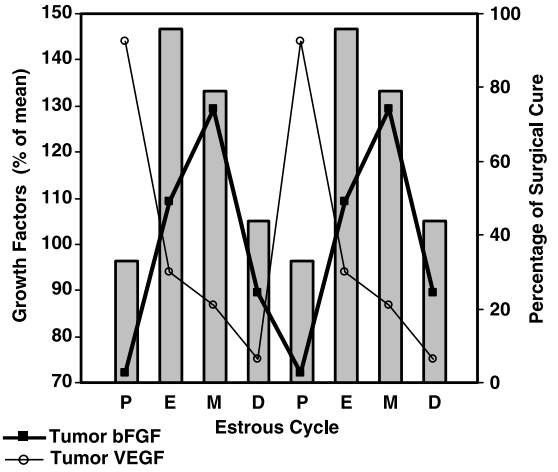

Figure 2. Double plot of the relationship between C_3H mammary tumor VEGF and bFGF protein concentration (as a percentage of mean values) plotted on top of surgical curability (*columns*) of this tumor across the estrous cycle. In proestrus tumor, bFGF is low and VEGF is high. In estrus, they cross one another and exist at their respective cycle mean values; they diverge again opposite to proestrus (high bFGF, low VEGF) in metestrus and then converge again during diestrus. Stages: *P,* proestrus; *E,* estrus; *M,* metestrus; *D,* diestrus.

phase, diestrus in C_3H mice ($P = 0.12$, Table 1). We also analyzed VEGF protein as a function of the fertility cycle in a totally different tumor model, methylcholantrenene A sarcoma in CD_2F_1 female mice. A similar and significant fertility cycle difference in tumor VEGF protein ($F = 3.42$, $P = 0.024$) was seen in this sarcoma tumor by immunohistochemical analysis, quantitated by image analysis (Fig. 3A). Sarcoma tumors showed higher VEGF protein levels in proestrus and diestrus, not unlike the pattern seen in the mammary tumors (Fig. 3B and C). Both of these tumors, by standard immunohistochemical analysis, stain positive for estrogen receptor-α, negative for estrogen receptor-β and positive for progesterone receptor (data not shown) and show estrous cycle dependence of tumor growth rates (1).

VEGF mRNA Levels. Mammary tumor VEGF mRNA levels did not vary prominently within the fertility cycle ($F = 1.3$, $P = 0.28$; Table 1). VEGF mRNA levels in tumors from oophorectomized animals were not significantly different from tumors obtained during diestrus ($P = 0.56$; Table 1).

VEGF Expression in Normal Mammary Tissue

VEGF Protein Levels. In contrast to the mammary tumor from the same host, VEGF protein levels in the normal mouse mammary tissue although, on average, some 3-fold higher in samples obtained from mice sampled during estrus (0.09 ± 0.03 pg/mg) versus proestrus (0.03 ± 0.002 pg/mg), did not vary statistically across the estrous cycle ($F = 2.4$, $P = 0.08$; Table 1). There was a significant decrease in VEGF protein levels in normal mammary tissue from oophorectomized animals compared with tissues obtained from low estrogen/progesterone diestrus animals ($P = 0.01$; Table 1).

VEGF mRNA Levels. In the normal mammary tissue, VEGF mRNA levels varied significantly across the fertility cycle ($F = 3.3$, $P = 0.03$). Two-fold higher message levels were found in mammary tissue samples obtained from mice in proestrus (0.35 ± 0.07) versus metestrus (0.17 ± 0.02, Table 1). VEGF message and protein each peaked in normal mammary gland at the same estrous cycle stage, proestrus, when both progesterone and estrogen levels are each high. VEGF mRNA levels in normal mammary tissue from oophorectomized animals were, however, not significantly different from breast tissue levels obtained from low estrogen state diestrus animals ($P = 0.48$; Table 1).

bFGF Expression in Mammary Tumors

bFGF Protein Levels. Immunoassay detection of bFGF protein in mammary tumors revealed that bFGF levels did not vary significantly across the estrous cycle ($F = 1.2$, $P = 0.34$; Table 1; Fig. 2). bFGF protein levels in tumors from oophorectomized animals are lower than in each cycle stage, but not statistically different from tumors obtained during diestrus ($P = 0.07$, Table 1).

bFGF mRNA Levels. bFGF mRNA levels are higher in mammary tumors from mice during the estrogen- and progesterone-rich proestrus stage compared with tumors from estrus, metestrus, or diestrus ($F = 4.4$, $P = 0.01$). Oophorectomy did not further diminish tumor bFGF levels compared with tumors from low-estrogen state diestrus animals ($P = 0.46$, Table 1).

bFGF Expression in Normal Mammary Tissue

bFGF Protein Levels. bFGF protein levels were higher, although not significantly at 0.05 error level, in normal mammary tissue obtained during the metestrus stage (the estrogen withdrawal portion of the cycle) compared with samples obtained at the other stages ($F = 2.6$, $P = 0.06$; Table 1). However, bFGF protein levels in normal breast tissue from oophorectomized and diestrus animals were significantly different ($P = 0.01$, Table 1).

bFGF mRNA Levels. Normal mammary tissue bFGF mRNA levels varied across the fertility cycle, with significantly higher levels in the mammary tissue obtained during estrus, the cycle stage associated with most frequent cure and lowest tumor growth rate, compared with those obtained at other estrous cycle stages ($F = 4.1$, $P = 0.01$; Table 1). bFGF mRNA levels in normal mammary tissue from oophorectomized animals were not significantly different from tissues obtained during diestrus ($P = 0.67$, Table 1).

VEGF, bFGF protein, and RNA Relationships between Tissues Across the Estrous Cycle

VEGF is a secreted protein; therefore, in tumor-bearing mice, VEGF concentrations are five times higher in serum than in breast cancer cells, and they are several hundred times higher in cancer cells than in normal mouse breast cells. The concentration of this molecule is modulated by the estrous cycle most prominently in cancer, where it peaks in proestrus. It, however, decreases precipitously to much lower values in estrus, when surgical curability is surest. Unlike in breast cancer, VEGF peaks in estrous in normal breast cells. bFGF concentrations in normal breast are six

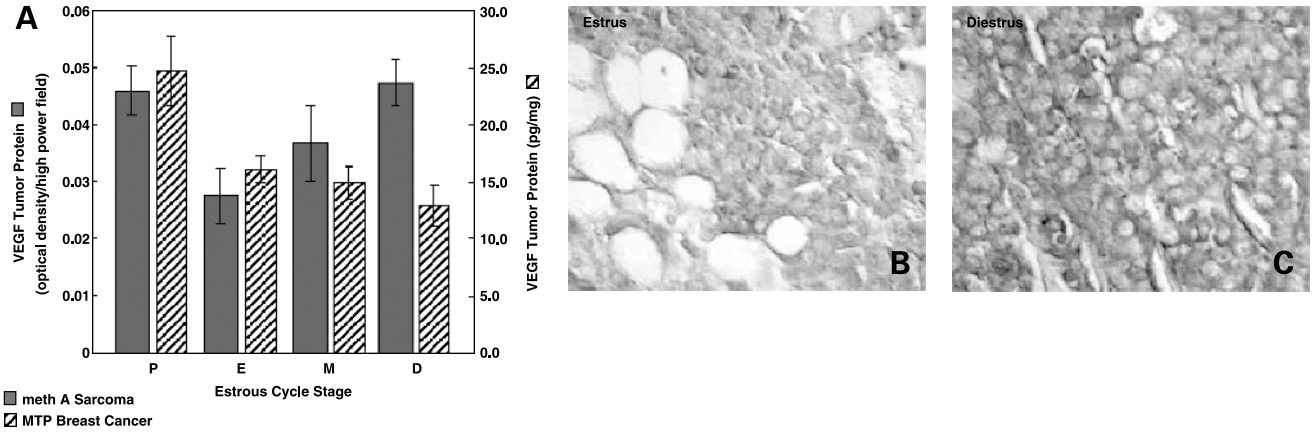

Figure 3. **A**, VEGF protein derived by immunohistochemical staining in methylcholantrenene A sarcoma tumors and enzyme immunoassay in tumor homogenates for MTP mammary tumors. VEGF tumor protein in both tumors (MTP breast cancer and meth A sarcoma) are each significantly different across the four estrous stages. Stages: *P,* proestrus; *E,* estrus; *M,* metestrus; *D,* diestrus. **B**, positively stained signals are mainly found in cytoplasm of the methylcholantrenene A sarcoma tumor cells which surrounds methyl green – stained nuclei (×40 light objective lens). **C**, the weakest VEGF signal occurs in tumors during estrus stage (*absorbance* = 0.022) and the strongest VEGF signal occurs within tumors during diestrus (*absorbance* = 0.074).

times higher than in breast cancer. The concentration of this protein is most prominently modulated by the estrous cycle in normal breast in which it peaks during metestrus. The VEGF and bFGF RNA both cycle in normal breast tissue. The bFGF RNA in breast cancer cells cycles prominently, whereas the VEGF RNA expression changes less robustly in these cancer cells throughout the estrous cycle.

Physiologic Endpoints

Mammary Tumor Vessel Density, Blood Content, and Capillary Permeability

To determine if the variations observed in tumor VEGF protein levels over the fertility cycle were associated with differences in tumor vascularity, we examined mammary tumor CD31–positive blood vessel density, blood volume, and capillary permeability in tumors obtained across the estrous cycle. There were no significant differences in the density of tumor blood vessels across the cycle (Table 1). The pattern of mammary tumor blood volume was higher in estrus (0.069 ± 0.013 mL blood/g tissue), lowest in proestrus (0.043 ± 0.015 mL blood/g tissue) but these differences did not, however, reach statistical significance ($P = 0.35$, Table 1). Mammary tumor capillary permeability, however, did vary with the cycle and was nearly 50% higher in diestrus, the cycle phase associated with the highest frequency of post-resection metastasis ($F = 3.9$, $P = 0.01$; Table 1; Fig. 4).

Circulating Sex Hormones

Estrogen and progesterone, expressed as percentage of the mean, are double-plotted along with surgical cure rates (frequency) at each estrous cycle phase in Fig. 1. Estrogen and progesterone concentrations changes occurring throughout the mouse estrous cycle have been well documented. We did not measure these hormones in our mice, however, we have retrieved published values (41, 42). The mean values and SEs for estrogen are (in pg/mL): proestrous, 10.3 ± 1.1; estrous, 4.2 ± 1.0; metestrus, 3.1 ± 0.7; and diestrus, 7.5 ± 1.3 (41). Progesterone levels at onset of activity are extrapolated from data published by Michael (42), proestrus, 60; estrus, 8; metestrus, 20; and diestrus, 8 (in ng/mL). Both estrogen and progesterone are highest during proestrus and decrease rapidly to low levels between proestrus and estrous. They remain at lower levels during metestrus and estrogen increases, whereas progesterone continues to decrease in diestrus (Fig. 1).

Tumor Growth Rate

Growth rates were calculated from serially assessed mammary tumor sizes of C_3H mice as a function of biological time (estrous cycle stage and cycle number) of measurement from our previous study (1). Standardized tumor growth rates vary significantly with fertility cycle phase (Table 1; Fig. 4). This cyclic effect is consistent across three separate studies of the C_3H mammary tumor. Average tumor growth rates across many estrous cycles are significantly different ($P < 0.001$) and are 2- to 3-fold higher in diestrus (636.2 ± 54 mm^3/d), as compared with other cycle stages (proestrus, 214.8 ± 30; estrus, 308.4 ± 38; metestrus, 276.1 ± 33 mm^3/d; Table 1). Estrous stage tumor growth across all cycles showed that tumor growth rate is highest in diestrus, and that cycle phase was associated with low surgical curability (Fig. 4). A similar dependence of tumor size/growth rate on fertility cycle is seen in the methylcholantrenene A sarcoma (1), which also shows fertility cycle dependence of tumor VEGF protein.

Estrous Cycle Pattern of Post-Resection Breast Cancer Spread

Two large, independent studies to determine the optimal time of breast cancer resection within the fertility cycle have recently been published (3). In these studies, 33% of mice (6 of 18) resected during proestrus remained free of metastases and were apparently cured, 96% of mice (25 of 26) resected during estrus were apparently cured, 79% of mice (11 of 14) resected in metestrus remained metastasis-free, and 44% of those (11 of 25) resected in diestrus were apparently cured (Table 1; Fig. 1). These

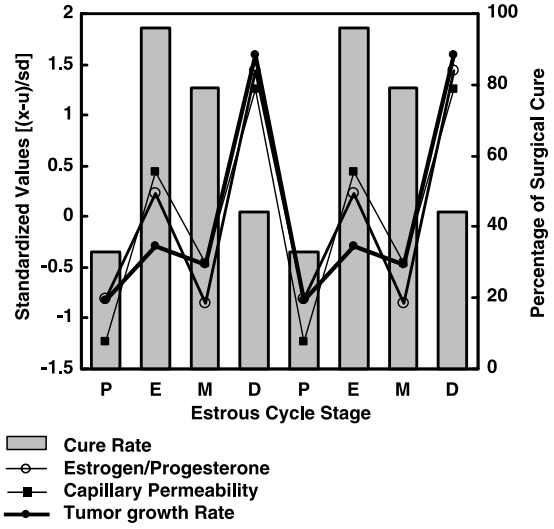

Figure 4. Covariation of the serum estrogen/progesterone ratio, capillary permeability of tumor vasculature, the rate of tumor growth, and surgical curability at each of the four estrous cycle phases. These coordinate relationships across the estrous cycle indicate that they are each tightly controlled by that cycle and perhaps by one another. The possibility that the estrogen/progesterone ratio controls the bFGF/VEGF ratio, which, in turn, influences capillary permeability and cancer growth rate, is raised by these covariations. Stages: *P,* proestrus; *E,* estrus; *M,* metestrus; *D,* diestrus.

surgical cure proportions are significantly different across the four estrous stages ($\chi^2 = 24.6$, $P < 0.001$). Oophorectomy also impacted cure with a cure frequency of 50% (5 of 10; $\chi^2 = 24.9$, $P < 0.001$; ref. 3).

The Relationships of Sex Hormone Concentrations and Angiogenesis Modulators to Capillary Permeability, Tumor Growth, and Post-Resection Cancer Spread throughout the Estrus Cycle

The ratio of serum estrogen to progesterone, tumor capillary permeability, and tumor growth rate covary throughout the cycle almost perfectly. Figure 4 shows that the highest serum estrogen/progesterone ratio, the highest tumor capillary permeability and the fastest tumor growth rate each occur in tumors during diestrus. Diestrus is that time within the cycle when post-resection spread is most likely. Estrus, the phase of the cycle when 96% of the mice are cured by resection, is associated with lower serum estrogen/progesterone ratios, lower tumor capillary permeability, and lower tumor growth rates.

Figure 5 shows that the ratio of tumor bFGF/VEGF protein also changes during each cycle. The highest ratios occur during estrus and metestrus when tumor spread after resection is least likely. This ratio in the tumor decreases during diestrus when surgical cure is least frequent and tumor growth rate and tumor capillary permeability are each highest.

Discussion

We have previously shown that the fertility cycle alters mammary tumor growth and post-resection spread in both mice and human beings (1–3). Others have shown how hormone concentrations vary throughout the estrous cycle (41, 42). During each estrous cycle, the concentrations of estrogen and progesterone peak concurrently in proestrus in response to pulsatile hypothalamic secretion of follicle-stimulating hormone, luteinizing hormone, and other hormones. Estrogen and progesterone decrease rapidly during estrus, after follicular rupture, when the ova are available for fertilization. These highly compressed murine cycle phases are roughly comparable to the luteal phase of the menstrual cycle. If fertilization does not occur, a new crop of estrogen-secreting follicles are built and estrogen increases in the absence of progesterone during metestrus and decreases again in diestrus. The adjacent estrous cycle phases of highest cure frequency (estrus and metestrus) are preceded by rapid declines of both estrogen and progesterone. The cycle phases associated with most frequent postsurgical cancer spread are those phases preceded by and associated with the most rapid increase of each of these hormones.

We now show that those cycle phases associated with high and/or rapidly increasing VEGF and low and/or decreasing bFGF are those associated with the highest risk of post-resection breast cancer spread, leaky tumor capillaries, and fastest cancer growth rate (diestrus and proestrus). We further show that these fertility cycle effects on tumor VEGF protein are not limited to mammary tumors, because we find similar cycle-dependent differences in a sarcoma.

Human breast cancer VEGF and bFGF message and protein expression patterns are known independently to

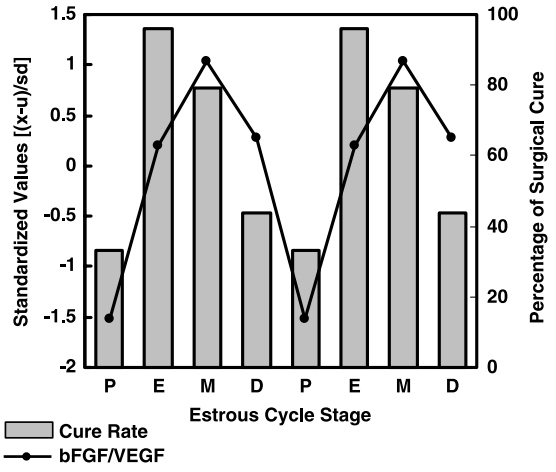

Figure 5. Relationship between the ratio of bFGF and VEGF protein levels within breast cancers and the likelihood of surgical cure of these tumors, as a function of when in the estrous cycle the tumors are resected. Those times of the cycle associated with the greatest bFGF/VEGF ratios are those same times associated with the highest rate of surgical cure/lowest metastatic potential: estrus (96% cure) and metestrus (79% cure). The estrous cycle stage associated with lowest probability of cure/highest metastatic potential, proestrus (33% cure), is associated with lower tumor bFGF/VEGF ratio. Stages: *P,* proestrus; *E,* estrus; *M,* metestrus; *D,* diestrus.

predict for metastatic cancer recurrence (15, 16, 22–24). Low levels of bFGF are associated with poor prognosis (15). High VEGF levels signal poor prognosis (22) and have been associated with high capillary permeability. In other mammary tumor studies, tumor VEGF protein levels have proven to be a more reliable predictor of tumor stage or aggressiveness than microvessel density or serum VEGF levels (25–27). We find that the highest VEGF levels occur during proestrus when cures are least frequent. VEGF levels are lower in the cycle stages associated with slower cancer growth and lower metastatic potential (estrus and metestrus). Buteau-Lozano et al. (44) have recently shown that VEGF can be transcriptionally regulated by estradiol and tamoxifen through an interplay of estrogen receptors-α and -β in transfected cancer cells. Manders et al. (45) have shown that high concentrations of VEGF predict breast cancer relapse and that progesterone receptor concentration is inversely correlated with both VEGF concentration and poor outcome. These data further support the connection among sex hormones, VEGF, and tumor outcome.

VEGF also influences other vascular events that are relevant to cancer metastasis. Recent work shows almost immediate nitric oxide–dependent effects of VEGF, mobilizing and remodeling preexisting host-derived latent vessels in growing tumors (46). VEGF also promotes adhesive interactions between the endothelium and tumor cells, white cells, and platelets. VEGF is likewise involved with the initiation of the deposition of a fibrin matrix necessary for promoting fibroblast and endothelial cell and successful tumor cell migration (22). In this capacity, VEGF could help establish the cellular foundation required for the boost of tumor growth we observe at each cyclical transition from estrus to metestrus (3), as well as the increased cancer spread following surgery in proestrus and diestrus.

Although tumor VEGF protein levels are modulated by the fertility cycle, VEGF RNA levels do not change markedly across the estrous cycle, which suggests that these sex hormone-induced increases in VEGF protein level occur through translational or posttranslational means. Differences across the fertility cycle/oophorectomy state in mammary tumor bFGF mRNA levels were observed. Highest bFGF mRNA levels were detected in tumor samples obtained during the proestrus stage of the fertility cycle, suggesting an estrogen- and/or progesterone-induced increase. This is further supported by the depression of tumor bFGF protein associated with oophorectomy. These estrous cycle changes in bFGF protein are relatively greater in normal breast than in breast cancer cells. bFGF concentrations are also several fold higher in normal breast. High levels of bFGF in breast cancer confer good prognosis as contrasted with VEGF (29). The balance between VEGF and bFGF and their rates of change during the cycle might be more important than the absolute levels of bFGF or VEGF. The ratio of bFGF and VEGF during the cycle shows the best covariation with surgical curability. The ratio of the bFGF to VEGF is highest in tumors resected during estrus and metestrus, when metastatic potential is lowest. The ratio is lowest among tumors resected during proestrus and diestrus, when resected tumors are most likely to spread.

We found no difference in serum VEGF levels across the fertility cycle or with oophorectomy state in tumor-bearing mice. This is in contrast to a published report demonstrating alterations in VEGF plasma levels in premenopausal breast cancer patients across the menstrual cycle, with lower serum VEGF levels in the luteal phase showing an obverse covariation with progesterone concentration (20). This discrepancy could be explained by the differences between the dynamics of the menstrual and estrous cycles between these two mammalian species and the serum half-lives of these proteins. The menstrual cycle is six times longer and hormone profiles, although similar, are not identical to those characterizing the estrous cycle. It may also be explained by the fact that we studied serum, not plasma. The contribution of blood platelets to serum VEGF levels is also significant. This may be an important source of species difference because blood cannot be obtained easily from mice (but could easily be obtained in women) without profound platelet activation (47).

There are interesting differences between RNA and protein patterns of both VEGF and bFGF between tumor and normal mammary gland. Although both the tumor and mammary gland show relatively high VEGF protein levels at or around the time of ovulation, only the normal mammary tissue shows fertility cycle–induced increases in VEGF mRNA. Conversely, both the tumor and mammary tissue show high bFGF mRNA levels at or around the time of ovulation, whereas fertility cycle variations in bFGF protein were only found in normal breast. In our system, peak VEGF protein levels were 200-fold greater in the tumor (proestrus) than in the mammary tissue (estrus), demonstrating a potentially pivotal difference in the role of VEGF in physiologic versus pathologic VEGF mediated processes. It is interesting to note that even though gene expression was several hundred fold greater in tumor than normal breast, both were equally well regulated by the estrous cycle. Conversely, peak bFGF protein levels were some 6-fold greater in the mammary tissue (metestrus) than in the tumor (metestrus), suggesting an important role for this growth factor in normal mammary gland physiology. Differences also exist in the ''kinetics'' of the fertility cycle–induced changes. VEGF protein levels were greatest in the mammary tumor during proestrus, whereas increases in normal mammary tissue VEGF protein levels ''shifted'' one stage and peaked in estrus. An identical ''shift'' in stages was seen with bFGF mRNA levels. These peak shifts suggest tissue-specific hormonal control of the same genes in subtly different host tissues (benign and malignant breast cells).

Estrous and circadian cycles each affect the host-cancer balance and are physiologically linked. Circadian coordination of estrous cycle events have long been proven (e.g., timing of ovulation to the activity stage). Lesioning the

SCN (central circadian clock) in female mice, rats and hamsters, greatly disturbs the reproductive cycle (48). Circadian *Clock* gene mutant mice more recently have been shown to have very abnormal estrous cycles (49). Vessel remodeling is essential for cancer growth and we have shown that cancer growth rate is modulated substantially by both the circadian and estrous cycles. We have shown that post-resection metastatic potential is influenced by the estrous cycle (3). VEGF tumor levels have been shown to correlate with metastatic behavior in a wide range of murine and human cancers (20, 22). Here we show that the estrous cycle modulates pro- and antiangiogenic molecules (VEGF and bFGF) within cancer cells. The ratio of bFGF and VEGF covaries with post-resection freedom from metastasis and the VEGF/bFGF ratio accurately predicts a high rate of post-resection metastatic cancer cell spread. Recent observations tie the molecular circadian clock within tumor cells to angiogenesis-modulated tumor progression, via circadian clock-mediated changes in VEGF and methionine aminopeptidase tumor cell gene expression (50, 51). Because the circadian clock regulates the estrous cycle, it is probable that the molecules modulating angiogenesis on the circadian scale may also be relevant to the tumor biology we are describing on the estrous cycle scale.

Discontinuous, intermittent, in fact, saltatory, growth seems to us characteristic of how biological systems organize growth. Traverse through the cell cycle is a necessarily saltatory process at the microscopic level; whereas saltation and stasis characterize human growth at the organismic level (52). Our data show that relevant negative and positive molecular regulators of angiogenesis and tumor capillary permeability, cancer growth rate, and post-resection metastatic cancer spread are each modulated by the mammalian fertility cycle. These covariations do not prove causation. Conclusions about causation await specific blockade of estrogen, progesterone, VEGF, and/or bFGF. If that specific blockade eliminates or multiplies the fertility cycle dependence of cancer growth and spread, and at the same time diminishes or increases average cancer growth rate and enhances or diminishes the post-resection breast cancer cure frequency, causation will be likely and might lead to the testing of periresection antiangiogenic therapies to improve breast cancer cure frequency. The presence of fertility cycling of VEGF in a sarcoma raises the possibility that the biology of other cancers, and the host-cancer balance, may also be affected by the female reproductive cycle.

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Brief communication

Sex cycle modulates cancer growth

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Key words: breast cancer, fertility cycle, chronobiology, estrous cycle, metastases, sex hormone

Summary

Hypothesis. Among premenopausal women, both post-resection metastatic potential and tumor growth rate are influenced by the menstrual cycle. There is strong support for the former in large retrospective studies of surgical resection timing within the menstrual cycle and the following experiments were conducted to critically evaluate the latter. *Methods*. We studied a transplantable breast cancer of C3HeB/FeJ mice (3 studies), and a transplantable methylcholantherene A induced sarcoma of CD2F1 mice (2 studies). We concurrently measured local cancer size and estrous cycle stage up to twice and at least once each day. There is a natural individual variability in the average length of normal estrus (3-1/2 to 7 days) cycle in mice. We assessed the effect of the cycle stage and cycle duration on tumor size. *Results*. We found identical estrous cycle stage coordination of cancer size, and identical effects of cycling frequency across all studies in each of these two tumors, both of which express both estrogen receptor alpha and progesterone receptor. Little or no change in cancer size occurs during proestrus (preovulatory phase) and estrus (periovulatory phase); tumor size increases several fold during diestrus (post-ovulatory phase); and the tumor shrinks partially as the next proestrus phase is approached. Across both mouse strains and tumor types, mice whose average cycle length is briefer (faster cyclers), have slower average tumor growth rate than those with longer cycles (slower cyclers) who have faster tumor growth rates. *Conclusion*. The virtually identical modulation of tumor size and cancer growth rate, in each of two very different transplantable cancers (one, classically sex-hormone-dependent, and the other, never previously recognized as hormone dependent) growing in two unrelated inbred mouse strains, indicates that the fertility cycle related host factors affect cancer size and growth rate. These *experimental* findings *suggest* that cancer cell proliferation of both breast and non-breast cancers in premenopausal women may be meaningfully coordinated by the menstrual cycle. *If this proves to be the case,* then any therapeutic strategy targeting proliferating cancer cells should be most effective against cancer of cycling women when given during the follicular phase of their menstrual cycles.

Introduction

Sex affects cancer outcome. The outcome for adult cancers affecting both sexes is superior in women. The younger the median age at diagnosis, the greater the female advantage [1,2]. Most childhood cancers show no advantage for female sex, until puberty. Interestingly, this female advantage is confined largely to cancers whose outcomes are influenced most by hematogenous tumor dissemination and those cancers treated primarily by tumor resection. These prominently include melanoma, epithelial carcinomas and sarcomas. Epidemiologic data connect menstrual cycle characteristics to cancer risk. The normal menstrual cycle duration varies from woman to woman between ~21 and ~35 days [3]. The risk of developing breast cancer varies with the average length of the menstrual cycle. The shorter the woman's cycle (the faster she cycles), the lower the breast cancer risk [4, 5].

Experimental carcinogenesis further supports a connection between cancer and the fertility cycle. Breast tumor incidence, tumor latency, and number of tumors induced by the direct carcinogen, N-methyl nitrosourea (NMU) and an indirect carcinogen, 7,12-dimethylbenzanthracene each depend upon the estrous cycle phase at the time of carcinogen administration. [6–8]. The frequency of tumor cell, H-*ras* proto-oncogene mutation in these NMU-induced tumors is likewise dependent upon the estrous cycle stage of NMU exposure [9].

The reproductive cycle affects the host-surgery-cancer interaction. When a transplantable mammary carcinoma

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of C3HeB/FeJ mice is resected after several weeks of growth, not every mouse is cured and some die subsequently from metastases, not unlike the human situation [10]. The timing within the fertility cycle of surgical resection of the breast tumor influences whether subsequent metastases occur [11]. An estrogen- and progesterone receptor-positive mammary cell line, derived from this primary tumor, also demonstrates this same estrous cycle dependence of surgical curability [12]. Estrous cycle coordination of host factors may be, in part, responsible for the cycle stage dependence of metastatic cancer spread. For example, splenocyte natural killer (NK) cell activity and interleukin-2 (IL-2) production, in tumor-free C3HeB/FeJ mice, vary rhythmically throughout each cycle. The periovulatory cycle stages associated with the lowest metastatic potential, demonstrate the highest NK activity and IL-2 production [13]. Women resected during the putative early luteal phase of their menstrual cycles have a 25% better chance of surviving breast cancer than those women whose breast cancers are resected in the follicular phase, during or nearer to monthly menses [14]. The dozen most complete retrospective studies indicate that optimal resection timing may enhance 10 year disease free survival by an average of 25% in absolute terms [15,16]. This is more than twice the benefit conferred by adjuvant chemotherapy. More than 40 subsequent retrospective studies of more than 10,000 women have largely, but not unanimously, supported these clinical observations [16]. A single ongoing prospective study has, so far, partially confirmed this biology [17,18].

We now present data in this same C3H murine breast tumor model, that predicted the importance of optimally timing breast cancer resection, showing that local tumor size changes rhythmically during each estrous cycle and that the cycling speed modulates average tumor growth rate. Furthermore, we demonstrate that this rhythmic cancer growth is not limited to breast cancer but also occurs in another mouse strain harbouring a chemically induced transplantable sarcoma.

Material and methods

Animals and tumors

The fertility cycle dependent growth characteristics of two different tumors in young cycling female mice were studied in five studies; three studies in an estrone binding, primary transplantable MTP mammary cancer in C_3HeB/FeJ female mice (n = 40, 120, 100 mice/study), [10] and two studies in a primary transplantable methylcholantherene A induced (meth A) sarcoma (E. Caswell, NY) in CD_2F_1 mice (n=126, 86 mice/study). Mice were maintained on a lighting schedule with 12 hours light alternating with 12 h of dark. Time of day (circadian time) is referenced to hours after light onset (HALO) with lights on at 0 HALO and light off at 12 HALO. Tumors cells were inoculated subcutaneously into the right flank and three dimensional tumor size (TS = length × width × height) was measured by calipers from the time of tumor appearance until sacrifice. Vaginal smears were obtained from mice at the time of tumor inoculation and at each tumor evaluation by gently flushing the vaginal os with saline and then fixing and staining the resultant cells with hematoxylin/thiazine (Diff Quick). Tumor measurements and estrous smears were obtained every 24 hours during the early activity phase (14 HALO) in three studies and every 12 h during early sleep phase (2 HALO) and again in the early activity (14 HALO) in two studies. In two of the C3H breast tumor studies, tumor-bearing mice were sacrificed at the time of final tumor measurement and the uteri were procured, trimmed of fat and wet weights determined. Tumors from these mice were assessed for wet-to-dry ratios as a function of estrous stage at the time of sacrifice by weighing tumors before and after dissecation of tissue in an 80 degree centigrade drying oven.

Fertitlity cycle determintations

Sequential estrous smears were evaluated for each mouse and classified, based upon cellular ratios, abundance of cornified epithelial cells, polymorphonuclear cells and non-cornified epithelial and the findings on the preceding and subsequent smears, into 1 of 4 stages (P, proestrus; E, estrus; M, metestrus; or D, diestrus) by standard criteria confirmed by previous correlations between vaginal cytology and uterine weight wet and a uterine proliferation marker [11]. Mice continue to cycle regularly in the presence of these tumors [19,20]. The ovarian cycle in the mouse ($\sim$ 4–5 days) and the woman (28 $\pm$ 7 days) are not strictly comparable throughout all stages. However in both, ultimate follicular maturation ends with mature follicular rupture and ovulation in response to FSH and then LH surges. In murine species during proestrus the LH/FSH surge is accompanied by a surge in progesterone (on top of rising estrogen) and these hormonal events are identifiable by vaginal cytology which demonstrates the proestrus to estrus phase transition at which time ovulation usually occurs.

Not all mice cycle at the same rate (variable cycle lengths) and groups of mice are not synchronized in the appearance of estrous cycle stages. Therefore to compare endpoints in mice at different estrous cycle stages, successive cycle numbers (cycle number 1, 2, 3, etc) and estrous stages within each of these cycles (P1, E1, M1, D1; P2, E2, M2, D2; etc) were assigned starting from the time of tumor inoculation until the last measurement. From these assignments, the total number of estrous cycles completed in a fixed time interval between the time of tumor inoculation and the last day of measurement (e.g cycling frequency), was calculated for each mouse by counting the successive appearances or transitions through the proestrous stage.

Protein immunohistochemistry

Tumors were fixed in 10% buffered formalin and paraffin embedded. The tissue sections, after deparaffinization and hydration, were (digested using proteinase K (20 ug/ml in PBS) for 20 minutes and washed in PBS twice (pH 7.2) for 5 minutes). Endogenous peroxidase activity was blocked by 3% H_2O_2 in PBS for 15 minutes. Slides were incubated in normal goat serum for 2 hours at room temperature. The primary rabbit polyclonal antibody against estrogen receptor (ER α, 1:400, ER β, 1:200) or progesterone receptor (PR, reactive with both A & B subtypes, 1:400) was applied to sections and incubated overnight at 4 °C. The secondary antibody (goat anti-rabbit IgG) and AB complex (Vectastain ABC kit, Vector Laboratory Inc., Burlingame, CA) were applied for 30 minutes at room temperature, respectively. Between incubations, slides were washed three times (5 min/each) in PBS. The color was developed by 3,3′-diaminobenzidine tetrahydrochloride (Peroxidase substrate kit DAB, Vector Laboratory Inc., Burlingame, CA). The sections were finally counterstained with Harris' hematoxylin (Sigma, St. Louis, MO). The same slides were stained without primary antibody as negative controls.

Statistical analysis

Variance among mean values, across more than two groups (e.g. 4 estrous cycle stages) were contrasted using one way analysis of variance (ANOVA) for repeated measures using the SPSS program. Tumor values are expressed as raw tumor volumes at each successive estrous stage and cycle number and as a percent of the mean of all tumor sizes for each fertility cycle (e.g. %mean tumor volume = TS from a mouse in P1 divided by mean all TS in cycle 1 × 100). Uterine weights are expressed as absolute wet weights at each fertility cycle stage and as the percent of the mean value across all stages. Analysis of tumor volume data against number of estrous cycles completed was performed using a repeated measures growth model with Proc Mixed (SAS version 8.02), where restricted maximum likelihood estimation procedure is used and that it considers the number of cycle group as a fixed effect. The unstructured covariance matrix was used. The effects of number of cycles completed, day, and the interaction of number of cycles and day were evaluated by F tests. Depicted values are the mean ± standard error for grouped data from individual mice.

Results

Not unlike the uterus, tumor size waxes and wanes throughout the fertility cycle

The fertility cycle stage of tumor inoculation did not affect the time to tumor appearance (palpable tumor) or the subsequent rate of tumor growth over time in the C3H breast tumor or meth A sarcoma (data not shown). Daily tumor size in both models increases in a typical sigmoid dependent pattern when fertility cycle stage is ignored. However, when C3H breast tumor size is serially assessed as a function of biologic time (estrous cycle stage and cycle number) of measurement, the influence of fertility cycle stage upon tumor growth is apparent by visual inspection of average tumor sizes (Figure 1A, Table 1). By grouping animal's tumor measurements according to each serial stage of each serially completed estrous cycle of each mouse, and examining average tumor sizes, as a function of cycle number and stage, it becomes obvious that tumor size waxes in metestrus and diestrus and wanes during proestrus and estrus. This cyclic effect is consistent across three separate studies of the C3H breast tumor. Virtually identical results are seen in two studies with transplantable methylcholanthrene A induced sarcoma (meth A sarcoma) in CD_2F_1 mice (Figure 1B, Table 1). These changes in tumor size with fertility cycle stage in each tumor type were seen when tumor measurements, along with vaginal smears, were monitored either during the early sleep phase (2 HALO) or during the early activity phase (14 HALO) of the 24 hour circadian cycle of the mice (data not shown). Therefore this estrous biology is present both in the activity and sleep phases of the daily cycle.

Analysis of raw tumor size by both cycle number (e.g. first, second, third, fourth, fifth) and estrous cycle stage within each cycle (P1, E1, M1, D1; P2, E2 etc.) by two way analysis of variance for repeated measures confirms the significance of this phase locked tumor growth in the C3H breast tumor and in the CD_2F_1 meth A sarcoma tumor models. Tumor sizes during each fertility cycle can also be expressed as percentage of the mean tumor size for that cycle in each of the studies, and then analyzed by one way ANOVA for the overall effect of estrous cycle stage (Table 1). In the C3H breast tumors, tumor sizes are 23–47% of cycle mean size during proestrous, increase gradually throughout both estrous and metestrous stages to a peak of 207–283% of the mean in diestrus, varying on average some 6.4 to 8.9 fold throughout the fertility cycle ($p < 0.001$). In the meth A sarcoma, tumor sizes are 50–59% of mean cycle size during proestrus, increase gradually throughout both estrus and metestrus stages to a peak of 149–185% of mean cycle size in diestrus, varying on average some 2.5 to 3.7 fold throughout the fertility cycle ($p < 0.001$). This rhythmic variation in tumor size follows the identical pattern in both tumors, with highest values in diestrus and lowest values in proestrus or estrus, although the magnitude of this cyclic effect is about twice as great in the mammary tumor. This cyclic tumor growth is analagous of the classically described waxing and waning of uterine size that occurs throughout each fertility cycle, secondary to the well described rhythmic sex hormone-driven changes in cellular proliferation, stromal proliferation,

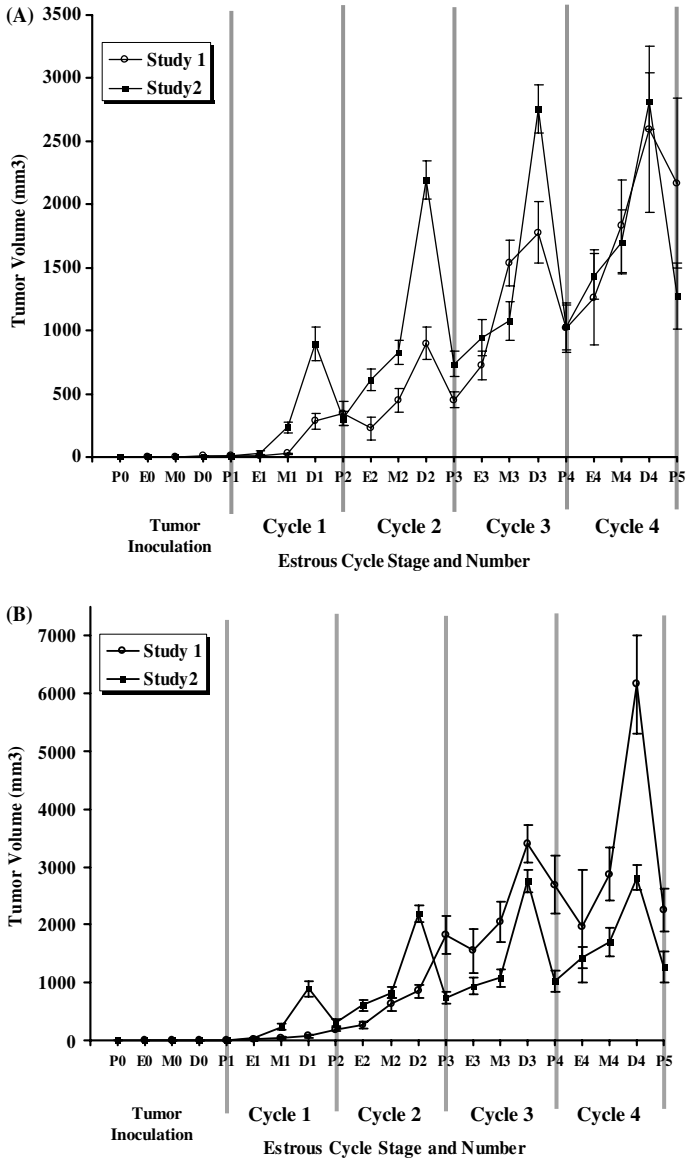

Figure 1. The effect of estrous cycle stage upon tumor size. Subcutaneous mammary tumor volumes in C_3HeB/FeJ female mice (A) and meth A sarcoma tumor volumes in CD_2F_1female mice (B) vary as a function of fertility cycle stage (P, proestrous, E, estrous, M, metestrous, D, diestrous) during each successive fertility cycle (cycle 0 or tumor inoculation to cycle 4) in two studies. Tumor sizes change rhythmically with fertility cycle stage in both tumor models with very little increase in tumor sizes during proestrous and estrous stages and large increases during metestrous and diestrous stages.

blood vessel number, size and blood content. In these C3H tumor-bearing mice, wet uterine weights in two studies, expressed as percent of mean cycle weight, vary 1.5 to 2.0 fold throughout the fertility cycle with highest values, as expected, in proestrus and estrus, and lower values in metestrus and diestrus (Table 1, $p < 0.01$). The pattern of cyclic change in tumor size is opposite to the pattern of the observed fertility cycle dependent change in wet uterine weights in these same tumor-bearing mice.

Analysis of wet-to-dry ratios of tumor weights as a function of estrous cycle stage at sacrifice (study 2 C3H breast tumor), failed to show significant estrous cycle differences ascribable solely to fluid content (proestrus 6.4 $\pm$ 0.3 estrus 6.7 $\pm$ 0.3) metestrous (6.6 $\pm$ 0.2) diestrus (6.5 $\pm$ 0.1)($p = 0.73$). These data indicate that large fluid shifts are not responsible for these very large tumor size differences but rather than these substantial size differences are more likely the result of coordinated bursts of tumor cell proliferation and stromal lattice to support these tumor cells.

Cycling frequency of the host modulates overall tumor growth rate

The normal menstrual cycle duration varies among women from 21 to 36 days [3]. Some mice also cycle faster than others with a range from 3 to 7 days [3]. We determined the effect of cycling frequency upon tumor growth. The faster the mouse cycles, the slower the tumor growth, and vice versa (Figure 2A and 2B). Average tumor growth is reproducibly slower in mice completing a greater number of cycles in a fixed span (e.g. 4 to 7 cycles in 12 to 14 days) while tumor growth is faster, nearly

Table 1. Effect of fertility cycle stage and cycling frequency upon tumor size and growth rate

Tumor Model	Fertility cycle stage						Cycling Frequency Daily Tumor Growth Rate
	Tumor size (% Mean of each cycle)						
	Proestrus	Estrus	Metestrus	Diestrus	Ratio of Change	*F, p*	*F, p*
C3H Breast Tumor							
Study 1	46.9 ± 7.5	37.8 ± 7.1	73.2 ± 7.9	219.5 ± 29.7	5.8	24.2, <0.001	5.4, 0.001
Study 2	23.4 ± 2.4	38.2 ± 3.6	72.8 ± 6.3	207.1 ± 13.6	8.9	86.3, <0.001	5.3, <0.001
5 cycles (n = 8)	88.85 ± 25.1	79.6 ± 31.0	99.6 ± 47.3	137.1 ± 30.5	0.0	NS	
4 cycles (n = 33)	69.3 ± 15.0	73.0 ± 14.3	71.0 ± 13.8	167.8 ± 21.2	2.4	8.8, <0.001	
3 cycles (n = 44)	56.1 ± 9.2	73.3 ± 11.6	69.5 ± 9.8	170.5 ± 15.7	3.0	18.8, <0.001	
2 cycles (n = 33)	43.5 ± 9.7	50.1 ± 9.8	70.8 ± 10.2	193.0 ± 18.8	4.4	27.3, <0.001	
1 cycle (n = 9)	4.4 ± 3.4	16.3 ± 6.7	71.5 ± 17.3	219.4 ± 32.6	50.0	18.3, <0.001	
Study 3	44.3 ± 6.5	50.4 ± 7.6	82.6 ± 9.8	282.7 ± 69.5	6.4	15.1, <0.001	4.9, 0.001
CD2F1 Meth A Sarcoma							
Study 1	50.22 ± 7.1	61.4 ± 14.9	105.2 ± 15.4	184.6 ± 33.6	3.7	9.4, <0.001	3.2, 0.043
Study 2	58.6 ± 4.2	76.1 ± 6.4	121.4 ± 17.1	149.1 ± 26.2	2.5	6.9, $p<0.001$	4.9, 0.001
Uterus	Uterine Wet Weight (% Mean of Cycle)						
C3H tumor bearing mice							
Study 2	150.1 ± 21.5	168.4 ± 5.7	118.6 ± 5.7	88.0 ± 2.4	1.9	41.6, <0.001	
Study 3	125.3 ± 11.0	107.0 ± 4.9	88.1 ± 4.6	104.1 ± 7.2	1.4	4.4, 0.006	

For each successive fertility cycle (cycle 1, 2, 3, etc), tumor volume at each stage within a given cycle (e.g. P1, E1, M1, D1, etc) is expressed as percentage of the mean tumor volume for all stages within that one cycle. Uterine wet weights at each fertility cycle stage have also been expressed as percentage of mean values across all stages. For study 2 in C3H breast tumor, the first line includes data from all mice and the subsequent lines include data from mice and the subsequent lines include data from mice completing different numbers of cycles (1–5) during the same time interval. Values are means ± SE. Analysis results (F, *p* values) from one way ANOVA for the effect of fertility stage upon tumor size, uterine wet weights and two way ANOVA for effects of cycling frequency upon daily tumor growth rates are listed.

double that rate, in mice completing fewer cycles (e.g. 3 or fewer cycles) in the very same span. These tumor growth rate differences are significant in both strains (C3H $F = 5.3, p < 0.001$; CD_2F_1 $F = 4.9, p = 0.001$).

In study two, we determined whether the effect of cycle stage on tumor growth was present among both slow and fast cyclers. Table 2 demonstrates that the effect of cycle stage on tumor size persists regardless of cycling speed.

Hormone receptor status of these tumors

Both the MTP mammary tumors and the meth A sarcoma tumors stain positively for estrogen receptor alpha, negative with estrogen receptor beta and positive for the progesterone receptor (data not shown) by standard immunohistochemical analysis.

Discussion

We have shown that the growth of two transplanted subcutaneous tumors, a spontaneously arising, sex hormone responsive, potentially metastatic breast tumor, and a chemically-induced locally aggressive sarcoma, grown in two distinct mouse strains, is virtually identically coordinated by the fertility cycle. These data indicate that the fertility cycle influence upon tumor biology and the host-cancer balance is not limited to tumors of breast or endocrine tissue origin and is thereby a phenomenon of more general significance. Because we have documented the presence of estrogen and progesterone receptors in both of these tumors, whether this cyclical behavior exists in ER negative and/or PR negative tumors remains to be determined. The fertility cycle stage dependent change in tumor growth is analogous to the estrous cycle dependent change in uterine size, but tumor size peaks at the opposite phase of the cycle in these same mice compared to uterine size. The speed of the estrous cycling also affects average tumor growth rate. Faster cyclers demonstrate twofold slower tumor growth than slower cyclers. These data are consistent with epidemiologic findings relating menstrual cycle length and breast cancer risk. Faster cycling is associated with the lower subsequent breast cancer risk [4, 5].

One hundred sixty-eight years ago, A.P. Cooper, who defined Cooper's ligaments of the breast, observed that breast cancer growth waxes and wanes regularly within the young women's menstrual cycle [21]. More than a century ago, G.T Beatson connected breast size and milk production to the ewe's 28 day fertility cycle [22]. When Beatson was made professor of surgery Glasgow, he acted

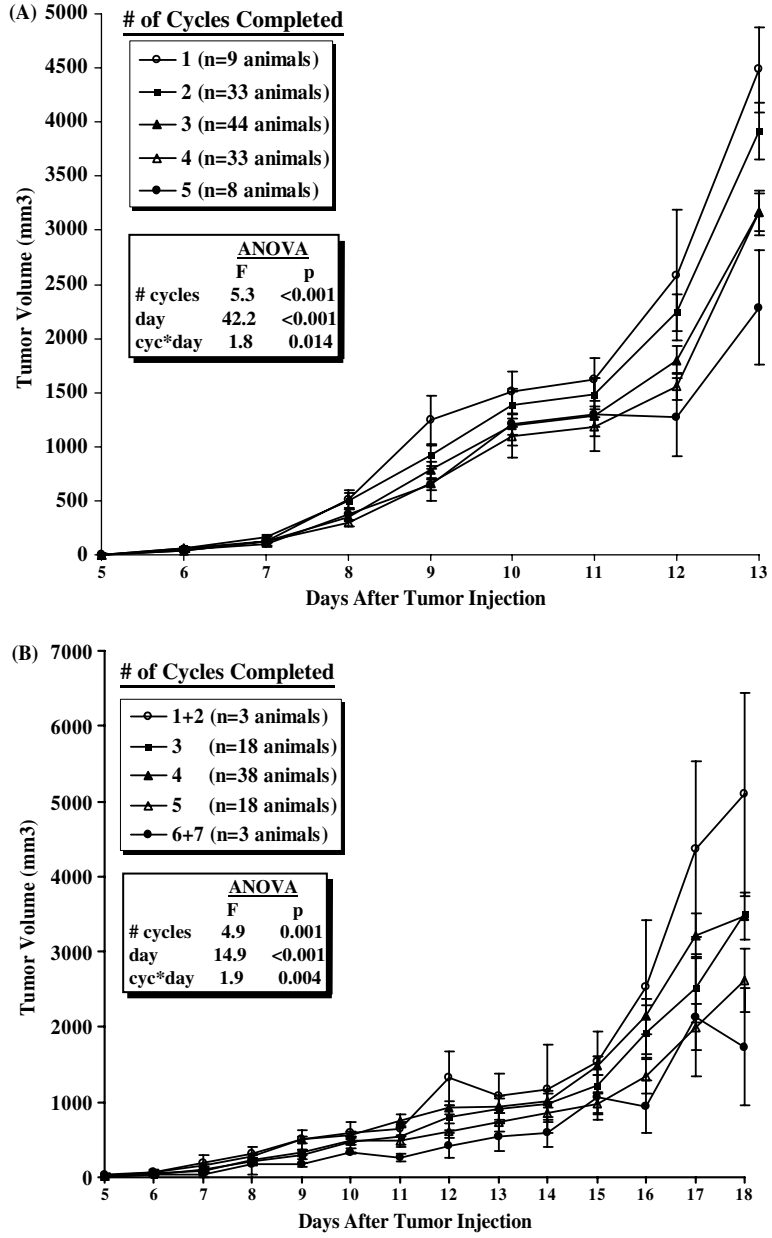

Figure 2. The effect of host cycling frequency upon tumor growth rates. Individual mice were classified by number of completed fertility cycles (1–7 cycles) in the same time interval and tumor growth rates are compared in each model of mammary tumors in C_3HeB/FeJ female mice (A) and meth A sarcoma tumors in CD_2F_1female mice (B). The number of fertility cycles traversed significantly affects tumor growth rates in both tumor models. Mice.

upon his observations of the connection between ovary and breast, by performing oophorectomy upon young women with lethal metastatic breast cancer. His reports were the first of many in the last century to document the fact that breast cancer in women can be controlled and even caused to remit entirely, following female castration [23]. In 1959 through the 1980's, Fisher and Fisher defined the biology of tumor dormancy and demonstrated how the resection of the primary tumor affects the biology of metastatic breast cancer spread [10, 24, 25]. More recently, we demonstrated, in cycling mice and women, that whether breast cancer spreads/recurs after resection depends upon when in the estrous or menstrual cycle the resection is done. Aggregate clinical data indicate that optimal resection timing, mid cycle and during the early luteal phase, gives a young woman as much as a 25% better chance for ten year disease free survival [15, 26].

Herein, we report prominent waxing and waning of tumor size and growth rate within the fertility cycle in mouse breast cancer, and a chemically-induced mouse sarcoma. These findings are entirely consistent with what Cooper reported in the breast tumors of young women. This cyclic cancer biology reflects the essentially intermittent or saltatory nature of growth, by nature and logic, a cyclical rather than linear or continuous process. This biologic growth pattern is not unique nor unprecedented. Growth studies in children using serial height determinations also provide support for periods of growth interspersed with periods without significant changes in height [27]. Further, in normal reproductive tissues (e.g.

breast, uterus), rhythmic periodic changes of cell proliferation and apoptosis are classic findings during each fertility cycle [28]. There is evidence that cellular proliferation in benign human neoplasms also change within the menstrual cycle [29]. Prominent daily rhythms in cellular proliferation have been well documented in murine and human cancer [30–32].

In summary, both fertility cycle stage and cycling frequency affect the growth rates of two different experimental cancers growing in two unrelated strains of female mice. If these findings, which are consistent with early clinical observation, are also clinically relevant, then, the effect of the menstrual cycle on cancer growth and post-resection cancer spread, may be a general one, not limited to breast cancer. Since it seems likely from these data that cancer cell proliferation and apoptosis as well as angiogenesis and stromal proliferation are each highly coordinated within each reproductive cycle, it is also *possible* that the effectiveness of therapeutic strategies which depend upon the expression of targets relevant to these processes *may be* dependent upon when in the reproductive cycle they are used.

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Review

Does surgery unfavourably perturb the "natural history" of early breast cancer by accelerating the appearance of distant metastases?

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Abstract

This historical perspective on breast cancer tells us how and why certain therapeutic eras have reached ascendancy and then declined. Therapeutic revolutions occur after a crisis develops when there is a general recognition that clinical interventions are not producing positive results predicted by the prevailing paradigm. The attitude of pre-modern surgeons was influenced by the very real possibility of doing more harm than good by operating upon women with breast cancer. Up until Halsted, the general consensus was clearly that, unless forced by the circumstances, surgical resection should be avoided for disease much more advanced than very early stage tumours (the cacoethesis of Celsus). Twentieth century progress in antisepsis, anaesthesia, and surgery changed this point of view. The first three quarters of that century saw more and more aggressive operations performed while the last quarter century reversed this trend, with reduction of the size of breast cancer operations based largely on the teachings of Fisher. A new crisis is upon us now in that trials of early detection have resulted in unexpected disadvantages to certain subgroups and there is previously unreported structure in early hazard of relapse, clinical data that suggests the act of surgery might accelerate the appearance of distant metastases. The explanation we propose that agrees with these results, as well as physicians of antiquity, is that surgery can induce angiogenesis and proliferation of distant dormant micrometastases, especially in young patients with positive nodes.
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Keywords: Surgery; Breast cancer; Metastases; Natural history

1. Introduction

In this paper, we concentrate on the natural history of breast cancer and the punctuated evolution of *conceptual models* to explain its behaviour [1–3]. From antiquity until the 18th century the subject of breast cancer was dominated by the philosophy of Aristotle and the therapeutic dogma of Galen that thought of breast cancer as an imbalance of the vital humours. The therapeutic consequence of this belief was purgation and bleeding to rid the body of a putative excess of *melancholia*. Despite this, tumour removal was a not exceptional therapeutic option (Galen himself excised "small" tumours and recommended excision through surrounding healthy tissue). However, a common belief was that a few favourable results, if any, could seldom be achieved by removal of small easily resectable lumps, while surgery was to be considered detrimental *quoad vitam* and *quoad valetudinem* for more advanced cases.

Celsus (30 BC–38 AD) established the first staging system of cancer. "First there is the cacoetheses, then

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carcinoma without ulceration, then the fungating ulcer... None of these can be removed but the cacoetheses; the rest are irritated by every method of cure. The more violent the operations the more angry they grow. Some use caustics, some burning irons, others remove the growth with the scalpel. After excision, even though a cicatrix is formed, it recurs, bringing with it the cause of death, whereas at the same time, most people, by using no violent methods to attempt the extirpation of the disease but only applying mild medications to soothe it, protract their lives, notwithstanding the disorder, to an extreme old age.'' This was relative of course to the life-expectancy in those times.

A first important change occurred in the 18th century, and was prepared by the crisis of the Galen system following new anatomy findings (Vesalius, Harvey) and the introduction of the microscopical inspection (Leeuwenhoek, Malpighi). Henry LeDran (1684–1770), a French surgeon, first challenged the dominance of the Galenic model. He stressed that cancer was a local lesion in the early stages and spread via lymphatics. LeDran observed that cure was much less likely when lymph nodes were involved. This breaking of anatomical boundaries signalled a stage unfavourable for intervention.

Velpeau (1856), a Frenchman, advocated bleeding, leeches, purgatives and emetics. He retained the Galenic ideas and used various drugs to destroy the humours. Velpeau is considered by some to be the first medical oncologist, Galenic though he was. He wrote: ''To destroy a cancerous tumor by surgical means is usually an easy matter and but little dangerous in itself; but the question arises, whether such a procedure affords a chance of radically curing the patient. This proposition remains undecided.'' He further states: ''The disease always returns after removal, and operation only accelerates its growth and fatal termination.''

James Syme (1799–1870), a Scotsman, condemned the practice of palliative procedures including purging; bleeding, the application of ointments, and limited surgery in the treatment of cancer. He stated: ''The only proceeding that deserves at all to be considered a remedy for cancer is removal of the morbid structure.'' This was a rather audacious statement considering that all of this was done without the availability of microscopic anatomy. Further, all surgery was performed without antisepsis or anaesthesia. Nitrous oxide was described in 1842 and ether was first demonstrated in 1846.

Schleiden (1838), a German, was among the first to appreciate the significance of the cell as a unit in plant structure, and Virchow, another German, considered to be the father of pathology, advanced the concept that any normal cell can become a cancer cell as a result of irritation. These concepts bolstered by microscopic examination of excised tissues led to a fuller understanding of the infiltrative and invasive nature of this disease.

Sir James Paget (1814–1899) first observed that proper seed and soil are necessary for cancer to grow and subsequently spread. He also had great respect for the limitations of surgical separation of seed from soil. Paget stated: ''We have to ask ourselves whether it is probable that the operation will add to the length or comfort of life enough to justify incurring the risk of its own consequences.'' Despite the lack of anaesthesia or asepsis, he had an operative mortality rate of only 10% in 235 cases of breast cancer. However, he believed the disease to be hopeless and stated: ''In deciding for or against removal of the cancerous breast, in any single case, we may, I think, dismiss all hope that the operation will be a final remedy for the disease. I would not say that such a thing is impossible; but it is so highly improbable that a hope of its occurring in any single case cannot be reasonably entertained.''

This pessimistic attitude was also voiced by Robert Liston (1794–1847): ''No one can now be found so rash or so cruel as to attempt the removal of the glands thus affected whether primary or secondary.''

In this pessimistic atmosphere, less rather than more surgery was considered by some to be prudent. Velpeau favoured thorough excision in preference to complete amputation. He stated: ''If the disease requires, the pectoralis muscle should not arrest us. The smallest shade of the disease must be taken away, if we are determined not to lose any chance of success. However, should there appear to be any necessity of interfering with the bones or resecting the ribs we must not deceive ourselves. The return of the disease is then inevitable and it would have been better not to have undertaken the operation at all.''

Hayes Agnew (1818–1892), of the United States, resorted to surgery solely for its moral effect. He believed that surgery actually shortened the life of the patient. He was most pessimistic and stated: ''I do not despair of carcinoma being cured somewhere in the future, but this blessed achievement will, I believe, never be wrought by the knife of the surgeon.''

A treatise by Gross of Philadelphia published in 1880 [4] provides a clear insight into the understanding of the disease in the era immediately before the developments in anaesthesia and antisepsis which allowed surgeons to attempt a radical cure of breast cancer. He describes a series of 616 cases, 70% of whom had skin infiltration on presentation which had ulcerated through in 25% of the patients. About 64% had extensive involvement of axillary nodes and 27% had obvious supraclavicular nodal involvement. Accepting that the meagre benefits of surgery seldom outweighed the risks in those days, he judged it ethical to follow the natural course of 97 cases who received nothing other than ''constitutional support''.

Gross' observations were useful for understanding the natural history of advanced local breast cancer. He describes how skin infiltration appeared an average 14

months after a tumour is first detected, ulceration appears on average 6 months after that, fixation to the chest wall after a further 2 months and invasion of the other breast if the patient lived on average 32 months after the lump first appeared. The average time for the appearance of enlarged axillary nodes was 15 months in those few cases that presented with an "empty" axilla to start with. About 25% of all these untreated cases exhibited obvious distant metastases within a year and 25% after 3 years with only 5% surviving more than 5 years.

Since then a number of different series of untreated breast cancer have been reported. For example Greenwood in 1926 [5] described a 6-year follow-up of 651 cases of untreated breast cancer with only 60 remaining alive at the end of this period. Daland in 1927 reported a series of 100 patients who were considered inoperable, unfit for surgery or who had refused the offer of surgery. The average duration of life was 40 months for the whole group, 43 months for those deemed operable at diagnosis and 29 months for those deemed inoperable [2].

The study that has attracted the most attention over the years was that of Julian Bloom published in 1968 [6]. His data came from the records of 250 women dying of breast cancer in the Middlesex Hospital Cancer ward between 1905 and 1933. Of this group, 95% died of breast cancer, but it should be noted that almost all of them presented with locally advanced or overt metastatic disease. The survival rates from the alleged onset of symptoms were 18% at 5 years, 0.8% at 15 years (remarkably, one person lived 16 years) with a mean survival of approximately two and a half years. The reasons given for withholding treatment are also worthy of note: old age or infirmity 35%, disease too advanced 30%, treatment refused 20% and early death the remainder. Together, these observations lead to the conclusion that uncontrolled breast cancer is lethal with most patients dying within a couple of years, but with many living with the disease for some years longer.

It would of course be inconceivable to suggest we study an untreated group today and the closest approximation we can find comes from a report of the Ontario cancer clinics between 1938 and 1956, just preceding the jump in breast cancer incidence in the developed world [7]. Close on 10 000 cases were analysed accounting for 40% of all new cases arising in the province of Ontario during this period. Amongst this group were 145 well-documented cases who received no treatment of any kind. Although, yet again 100 of these cases were untreated because of late stage of presentation or poor general condition, the rest were unable or unwilling to attend for treatment. A careful note was made of the date the patient first became aware of the lump from which point survival rates were computed. The 5-year survival from first recorded symptom was 35%, with a median survival of 47 months. The most surprising figure was a near 70% 5-year survival for the small group presenting with localised disease.

This then raises the inevitable question, is carcinoma of the breast inevitably a fatal disease if neglected? This question is almost impossible to answer with confidence although hinted at by anecdotal evidence. However, the best documented in the literature was reported by Steckler and Martin in 1973 [8]. They described a 38-year-old woman with histologically proven cancer who refused surgery and was then followed up for 20 years before consenting. We will never know how many of the cases we see in our daily practice carry such a favourable natural history.

2. The influence of surgery on the natural history of breast cancer

From the popularisation of the classical radical mastectomy at the very end of the 19th century [9] until about 1975 almost all patients with breast cancer, of a technically operable stage, were treated with modifications of the radical mastectomy. To those without commitment to a prior hypothesis, this allowed for new insights about the nature of the malignant process. Before considering this matter, it is worth revisiting the conceptual model that allowed the radical operation to reign supreme for 75 years.

In about 1840, Virchow described a revolutionary model of the disease building on the development of microscopy and post-mortem examinations of the cadavers of breast cancer victims [10]. He suggested that the disease started as a single focus within the breast, expanding with time and then migrating along lymphatic channels to the lymph glands in the axilla. These glands were said to act as a first-line of defence filtering out the cancer cells. Once these filters became saturated the glands themselves acted as a nidus for tertiary spread to a second- and then third-line of defence like the curtain walls around a medieval citadel. Ultimately when all defences were exhausted, the disease spread along tissue planes to the skeleton and vital organs.

So convincing were these arguments and so charismatic their chief proponent, the Halsted operation was adopted as default therapy all round the world. At this perspective we are entitled to ask to what extent did the radical operation add to the curability of the disease and what can we learn about the nature of the beast by its behaviour following such mutilating surgery? We can also add a third question concerning human nature and our unwillingness to see facts "which almost slap us in the face" ("It is now, as it was then, as it may ever be, conceptions from the past blind us to facts which almost slap us in the face" – WS Halsted 1908) [11,12].

Unfortunately, only 23% of patients treated by Halsted survived 10 years [11]. The natural response to this

failure was even more radical surgery. Internal mammary lymph nodes that received about 25% of the lymphatic drainage of the breast were not removed in the 'complete operation', but included in the super radical operations that followed or in the extended fields of radiation after surgery.

Retrospective studies indicated that more radical operations improved survival [13]. However, in randomised trials that followed later, no benefit could be demonstrated [14,15]. Thus, even when the tumour seemed to have been completely 'removed with its roots', the patients still developed distant metastases and succumbed: 30% of node-negative and 75% of node-positive patients eventually dying of the disease over 10 years when they were treated by radical surgery alone [16] and with no evidence of "cure" if patients were followed up for 25 years [17]. In this latter seminal study by Brinkley and Haybittle, a group of over 700 breast cancer patients, treated by radical surgery alone and followed up for 25 years, steadily continued to demonstrate an excess mortality compared with an age-matched population.

3. The biological revolution of the late 20th century

Thinking began to change with Fisher. Prompted by the failures of radical operations to cure patients of breast cancer, Fisher proposed a revolutionary hypothesis that rejected the mechanistic models of the past [18]. He postulated that cancer spreads via the blood stream even before its clinical detection, with the outcome determined by the biology of tumour–host interactions. Based on this concept of 'biological predeterminism', he predicted the following:

(A) The extent of local treatment would not affect survival; and (B) systemic treatment of even seemingly localised tumours would be beneficial and might even offer a chance of cure.

Several pioneers in the field set up randomised clinical trials to test these hypotheses culminating in a series of world overviews [19]. Although the "Fisherian" doctrine is now taken as 'proven', we must accept that the proof is more in principle rather than in cure. The benefits from systemic therapy are modest, with a relative risk reduction in breast cancer mortality of approximately 25% overall, which translates to approximately 10% in absolute terms. As regards the extent of local treatment, many randomised trials have tested less versus more surgery with or without adjuvant radiotherapy.

A recent world overview of these trials [20] concluded that more radical local treatment, surgery or adjuvant radiotherapy does not have any influence on the appearance of distant disease and overall survival with one caveat (*vide infra*). This is in spite of the increase in local recurrence rates with less radical local treatment, i.e., although radical surgery or postoperative radiotherapy had a substantial effect on reducing local recurrence rates, it did not improve overall or distant disease-free survival.

The one exception to the theory of predeterminism might be the "success" of the trials of mammographic screening [21]. From this it might be concluded that 25% of breast cancer deaths in women aged 50–69 years could be avoided if caught "early" at a sub-clinical stage. Forgetting the arguments about the scientific reliability of these studies [22], this still only accounts for approximately 12% of incident cases, i.e., failing those cases in women under 50 years or over 70 years.

Even in the world overview there is one finding that was not completely in keeping with Fisher's doctrine of biological predeterminism. Radiotherapy does actually reduce the breast cancer-specific deaths by approximately 3% – only to be counterbalanced by the increased mortality from late cardiac complications in those patients with cancer in the left breast because of radiation damage to the heart. More recently, two randomised-controlled trials evaluated the benefit of postoperative radiotherapy after mastectomy for tumours with a poor prognosis. The radiotherapy techniques in these two studies minimised the dose to the heart. Not surprisingly, there was a reduction in local recurrence rates, but there was also an improvement in the overall 10-year survival rates – 9% [23] and 10% [24].

3.1. Adjuvant systemic therapy has only a modest effect on survival

The development of adjuvant systemic therapeutic regimens was based on the kinetics of tumour growth and its response to chemotherapy in animal models [25]. However, the early clinical trials predicted a large benefit and were consequently underpowered to detect the modest 'real' benefit. Consequently, there was considerable confusion, with the positive results of some of the early trials being contradicted by negative or equivocal results of others. However, the overview analysis confirmed that adjuvant systemic therapy can in fact be beneficial [19]. It is the magnitude of benefit that is disappointingly modest – an absolute benefit of a maximum of 12% in high-risk premenopausal individuals and of 2% in equivalent-risk postmenopausal individuals is much smaller than that anticipated from the experimental models.

The next step taken by medical oncologists was very similar in attitude to that taken by surgeons only a few decades ago, if a little does not work then try a lot! This approach was bolstered by the excellent rate of long-term cure achieved in haematological malignancies. In addition, tumour cell lines showed a log-linear dose response when exposed to alkylating agents [26,27].

Needless to say the high-dose chemotherapy with bone marrow rescue was a failure and the least said about this sorry episode in the history of breast cancer

the better, yet there may be lessons to learn from the failure of this approach.

3.2. When does a primary tumour seed its secondaries?

If we believe that once a primary tumour gains access to the vasculature it starts seeding metastases in a linear or exponential manner, it should be expected that because a larger tumour has been in the body for a longer time, and therefore has had access to the vasculature for longer than smaller tumours, a much higher percentage of patients with larger tumours should present with metastases. This is true to some extent with regard to lymphatic metastases, i.e., there is a correlation of number of involved lymph nodes with the size of the primary tumour. However, this relationship is far from linear. Thus, there are small or even occult tumours that have several involved lymph nodes, while many large tumours are found not to have metastasised to the axilla. This discrepancy becomes even more apparent when we consider distant metastases. It would be expected that the proportion of patients presenting with distant metastases would be higher for those with larger tumours as opposed to those with smaller tumours. Nevertheless, in real life a patient presenting with a primary tumour along with distant metastases is uncommon, however large the tumour. In fact, the percentages of patients that present with symptomatic metastases is 0%, 3% and 7% in stages I, II and III of the primary tumour, respectively [28]. However, when you look at the incidence of metastases in these same groups 18 months after their primary diagnosis and therapy, there is a clear correlation of primary tumour size with the proportion of patients experiencing distant relapse. (Approximately 5% for stage I and 25% for stage III.)

How can this be explained without challenging the linear model of breast cancer spread? One explanation would be that although the number of metastases that are seeded by the primary tumour would be linearly related to the tumour size and biological aggressiveness, the clinical appearance of metastases is triggered or accelerated only after the primary tumour has been disturbed or removed. This conclusion may logically derive from a consideration of the pessimistic experiences of ancient surgeons we presented in previous sections. It also is the result of very modern day science using computer simulations to analyse an unexpected bimodal hazard rate of relapse for patients treated only with surgical excision of primary breast tumours. Hazards are calculated by dividing the number of events in a particular time-frame by the number of patients at risk of having those events at the start of the period. This is an important way of looking at data because it emphasises when adverse events occur rather than just the cumulative result. Since no one lives forever, including breast cancer patients, when the increased risk for recurrence and death occur is more important than the overall risk. We show in Fig. 1 relapse data from the Milan series.

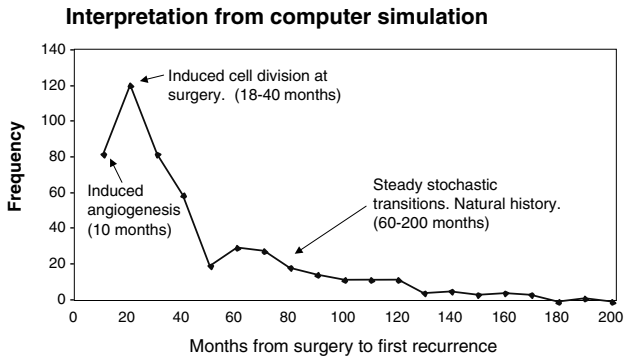

Fig. 1. These are relapse data from 1173 otherwise untreated early stage breast cancer patients with 16–20 year follow-up. There is a sharp peak at 18 months, a nadir at 50 months and a broad peak at 60 months with a long tail extending to 15–20 years. Patients with larger tumours more frequently relapse in the first peak while those with smaller tumours relapse equally in both peaks. Similar patterns to the Milan data can be identified in some but not all disease-free survival [52] and hazard of relapse [53] databases for untreated patients. Based on a computer simulation, breast cancer growth often includes periods of temporary dormancy. The second peak is the natural history of the disease. These relapses result from steady stochastic transitions from single cells (dormancy half-life of 1 year) progressing to an avascular micrometastasis (dormancy half-life of 2 years) to a growing lesion that eventually becomes detected as a relapse. The first peak is too sharp to be the result of steady stochastic transitions. Some breaking of dormancy had to occur at surgery to explain the first peak.

Naumov and colleagues [29] has observed dormant, but viable, single cells in a breast cancer animal model and Klauber-DeMore [30] has observed small dormant micrometastases and growing larger micrometastases in human breast cancer. Folkman and colleagues [31] have reported many examples of dormant micrometastases in animal models. Within the dormant micrometastases there is balance between growth and apoptosis. There are known factors that inhibit angiogenesis and other factors that stimulate angiogenesis. To maintain a dormant state, inhibiting factors dominate locally. If stimulating factors are increased or inhibiting factors are reduced, the dormant condition can no longer be maintained.

It is well documented in the Lewis-lung model that removal of the primary tumour will reduce angiogenesis inhibitors and it is known that after surgery a sharp spike in angiogenesis stimulators and growth factors occurs to aid in wound healing. Thus, it is not surprising that tumour angiogenesis and proliferation result after surgery to remove a primary tumour. Therefore, a likely trigger for 'kick-starting' the growth of micro-metastases, could be the act of surgery itself.

The first peak occurs at the same time, whether the tumour was at stage I or stage III. It is only the amplitude of the peak that changes with stage, the later the stage the higher is the peak, but the timing of the signal remains the same.

These phenomena suggest a non-linear dynamic model for breast cancer, which, like a chaotic system, is exquisitely sensitive to events around the time of diagnosis. It suggests that surgery could be responsible for accelerating the clinical appearance of metastatic disease. However, a randomised trial of surgery versus no surgery to prove this would no doubt be judged unethical in the absence of systemic therapy. Nevertheless, such a model is fortuitously available in the setting of randomised trials of mammographic screening [32].

Thus, with this new perspective we come back to discussing the trials of mammographic screening. In these trials, surgery is delayed in the control group by approximately 18–24 months (lead-time) so that the first few years offer the comparison between no surgery in the control arm versus surgery in the screened arm. Later years offer the comparison between "late" surgery in the control arm versus "early" surgery in the screened arm. In a meta-analysis of screening trials for breast cancer, it was found that in women under the age of 50 years, there is an early *excess* mortality in the third year. In women 50 years and above, there is no year with a significant excess mortality. Since the time between relapse and death in breast cancer is approximately 2 years, it is reasonable to conclude that the timing surgical-stimulated proliferative wake up and angiogenesis triggering for premenopausal node-positive patients could account for the excess mortality in the 3rd year of the trials.

Clearly a new model for breast cancer is needed that takes into account the fine dynamic balance between the tumour and the host, including various autocrine and paracrine factors which influence proliferation, apoptosis and angiogenesis.

4. A new model to explain the natural history of breast cancer

Taking all of this data into account we would like to develop a new model to explain the natural history of the disease which in addition to explaining the success of the Fisherian model of "biological predeterminism" also explains the clinical observations from antiquity or that fail to fit neatly into the contemporary early detection paradigm.

The conclusion that more surgery is better is similar to the conclusion that earlier detection/earlier therapy is better. However, this linear thinking has not served as well. The reaction of Halsted's disciples was simply to assume that surgery had to encompass a greater field. The reaction of the mammographic screening community has been identical calling for earlier and more frequent examinations. Neither radical surgery nor earlier screening-induced surgery are free of harm. This linear thinking has done more harm than good. This is because the host–cancer–surgery interaction is not linear.

First of all cancer should be seen as a process, not a morphological entity [33]. Individual cancers, while likely to originate from single cells, are constantly adapting to the local environment. There is no single substance or metabolic defect that is unique to cancer. Clonality, previously considered a hallmark of cancer, is neither always demonstrated in malignancy nor restricted to it [34]. The cancer cell is largely normal, both genetically and functionally.

The malignant properties are the result of a small number of genetic and/or environmental changes that have a profound effect on certain aspects of its behaviour. The three main processes of cancer (growth, invasion and metastasis) have their equivalents in normal tissues. Most cancers are diagnosed by virtue of their morphological or histochemical similarity to the tissue of origin. At the genetic level, with the exception of deletions, all necessary information is preserved, and the defective portion of DNA is relatively small. The key processes of malignancy are genetically controlled by the under- or over-expression of normal genes and their products that normally serve essential cellular functions such as the response to wounding. In addition, pathological and autopsy studies have suggested that most of the occult tumours in breast (and prostate cancers) may never reach clinical significance [35,36].

Demicheli and colleagues [37] have also argued that a continuous growth model of breast cancer fails to explain the clinical data. The continuous growth model yielded tumour sizes too large to be missed at the preceding negative physical examinations, and required growth rates are significantly lower than those consistent with clinical data. As mentioned before, the continuous growth model also fails to explain the biphasic recurrence pattern seen when hazards of recurrence are plotted for every year after diagnosis.

The new model [38,39] is based on the concept of tumour dormancy/latency, both in the preclinical phase within the breast and later with the micrometastases that seed in the early phase of the natural history of the disease, once the primary focus has developed its microvasculature. The latter remain dormant until some signal, perhaps the act of surgery or other adverse life-event stimulates them into fast growth.

Single viable cells may remain dormant for some time and may be induced to proliferate by environmental factors. Groups of cells without angiogenic potential can grow, but remain small (up to 10^5 or 10^6 cells). The metastatic focus may grow quickly if (i) a subset of these cells switch to an angiogenic phenotype and/or (ii) the inhibition of angiogenesis is removed. The model suggests that the metastatic development of unperturbed breast cancer is a sequential evolution from a non-proliferative to a proliferative state and from a non-angio-

genic to an angiogenic state, with stochastic transitions from one state to the next.

This model may explain the early peak of hazard function for local and distant recurrences in resected cancer patients by combining with the natural metastatic development of unperturbed disease surgery-driven proliferative wake up induced through growth stimulating factor(s) [40] (''the Fisher effect'') with the angiogenic signal following surgery (''the Folkman effect''). It also correlates well with the findings of a modest benefit after adjuvant systemic chemotherapy.

We can now add a new mathematical model to the biological model described above [41]. Breast cancer is like a complex organism existing in a state of dynamic equilibrium within the host, the equilibrium being very precarious and close to a chaotic boundary. Furthermore, the mathematics to describe the natural history of these ''organisms'' invokes non-linear dynamics or chaos theory. This model is the first attempt to apply the new mathematics of complexity to make predictions about the factors influencing the natural history of breast cancer, that might one day provide a therapeutic window.

Central to the understanding of this model is the pioneering work of Folkman on tumour angiogenesis [42]. As we know, solid tumours cannot grow beyond 10^6 cells or approximately 1–2 mm in diameter in the absence of a blood supply [43,44]. The initial prevascular phase of growth is followed by a vascular phase in which tumour-induced angiogenesis is the rate-limiting step for further growth and provides malignant cells direct access to the circulation [45].

In addition to the importance of the microvasculature, we can also visualise these microscopic foci as existing in a 'soup' of cytokines, endocrine polypeptides and steroids, with cells interacting with each other and with the surrounding stroma, interpreting competing signals directing the cancer cells in the direction of proliferation or apoptosis. Such complexity cannot be modelled by linear dynamics, or even a full understanding of the complete catalogue of genetic mutations at the cellular level, because the critical events of multiple cell-to-cell interaction require a thorough understanding of epigenetic phenomena.

What we now have is a new model of the disease that owes its genesis in part to the interpretation of the results of natural history databases or clinical trials by way of hazard rate plots rather than Kaplan–Meier curves. We can now see a new signal appearing against background noise, that challenges the assumption of linear dynamics in favour of non-linear mathematics or chaos theory [46]. This ''signal'' is the early peak of hazard for relapse that follows surgery within 48 months, whereas the stretched flatter curve thereafter might be the ''echo'' of the natural history of breast cancer left unperturbed by surgical interference.

If that is true then the act of wounding the patient creates a favourable environment for the sudden transfer of a micrometastasis from a latent to an active phase.

We must refocus on the host–cancer balance. We believe that careful reconsideration of both the therapeutic and deleterious effects of the wounding associated with breast cancer resection is in order. Breast cancer and the women who bear it comprise a complex system. The dynamics of the system are not linear. The entry into this complex system by any potentially therapeutic intervention could have very different outcomes depending upon the conditions of the complex dynamic host–cancer relationship at the ''time'' of the intervention. For example, timing of surgery within the menstrual cycle is very probably an important factor regulating surgery-induced angiogenesis for premenopausal node-positive patients [47,48].

The therapeutic consequences of the new models are almost self-evident. The intervention that suggests itself would be anti-angiogenic, and the timing of the intervention would be preoperative, so that at the time of surgery the system is primed to protect against sudden flooding with angiogenic signals. Indeed, some of the success attributed to adjuvant tamoxifen or chemotherapy might be a result of their anti-angiogenic potential rather cytostatic/cytocidal effects [49].

Assuming we can protect the subject from the first peak of metastatic outgrowth, we will then have to monitor her with extreme vigilance. By the time the metastases are clinically apparent it is perhaps too late, therefore monitoring the patient with tumour markers and reintroducing an anti-angiogenic strategy at the first rise might prove successful [50].

In the meantime, we can continue to add additional layers of complexity to the simulations of our mathematical model, to help develop alternative strategies for biological interventions to maintain the disease in equilibrium until nature takes its cull in old age [51,52].

Conflict of interest statement

None declared.

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Does surgery induce angiogenesis in breast cancer? Indirect evidence from relapse pattern and mammography paradox

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KEYWORDS
Angiogenesis;
Breast cancer;
Mammography paradox;
Surgery;
Dormancy;
Early detection

Abstract A significant bimodal relapse hazard pattern has been observed in two independent databases for patients untreated with adjuvant chemotherapy. This implies there is more than one mode of relapse. The earliest and most closely grouped relapses occur 8–10 months after surgery for young women with node-positive disease. Analysis of these data using computer simulation suggested that surgery probably instigated angiogenesis in dormant distant disease in approximately 20% of cases for premenopausal node-positive patients. We explore if this could explain the mammography paradox for women aged 40–49: an unexplained temporary excess in mortality for the screened population compared to controls. Calculations based on our data predict surgery-induced angiogenesis would accelerate disease by a median of two years and produce 0.11 early deaths per 1000 screened young women in the third year of screening. The predicted timing as well as the magnitude of excess mortality agree with trial data. Surgery-induced angiogenesis could account for the mammography paradox for women aged 40–49 and the bimodal relapse hazard pattern. According to the proposed biology, removing tumors could remove the source of inhibitors of angiogenesis or growth factors could appear in response to surgical wounding. While this needs confirmation, this could be considered when designing treatment protocols particularly for young women with positive nodes. It reinforces the need for close coordination between surgical resection and ensuing medical intervention. Women need to be advised of risk of accelerated tumor growth and early relapse before giving informed consent for mammography.
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Introduction

Breast cancer is a worldwide major health concern. While there have been reductions in mortality in recent years, progress is far too slow. In the US in 2005 it is estimated that there will be 212,930 new cases of breast cancer and 40,870 deaths from the disease.[1] Therapy has proven to be only partially effective in reducing death rates with little optimism until recently that major improvements are possible. The great hope for immediate meaningful reduction in breast cancer mortality was early detection, which is known to facilitate the discovery of breast tumors at a smaller size and with fewer positive nodes. The probability of cure for a 1-cm or smaller tumor and no lymph nodes involved is approximately 90%. With the reasonable probability that screening would detect more and more cancers in that or similar very early states, it was expected that mammographic screening would result in a major reduction in breast cancer deaths.

To avoid a bias, analyses are done based on invitation to screening rather than those who are actually screened.[2] When we discuss screening vs. controls in this document, the proper interpretation should be invited to be screened vs. controls who are not so invited.

As reported by eight randomized trials of breast cancer screening initiated between 1963 and 1980, women aged 50–59 who are screened have an early appearing 20–30% mortality advantage compared to unscreened control subjects. However, when women aged 40–49 years are screened, there is either no advantage or a slight disadvantage for the first 6–8 years of all trials. After that, an advantage begins to appear.[3–12]

When these disturbing results were first reported, a mammographer was quoted to say: ''You start screening and you expect to provide a benefit, and suddenly people die at a higher rate. Now, hold it, we're not going out and killing women. This demands an explanation''.[13] Pursuing this line of thought, if more women died of breast cancer in the screened arms than in the control arms, the trials themselves must be spurious.

Since these trials covered the full range of the cancer experience from randomization of a great many (apparently) healthy subjects to ultimate death from cancer or (much more likely) from any other cause, there are many opportunities to introduce bias or other errors. It was easy to criticize these trials. These data are, however, all we have to modulate our biases.

Following the National Institutes of Health Consensus Development Conference on Breast Cancer Screening for Women Ages 40–49, where all trial data were presented, two different and contradicting reports were published.[14] A consensus panel voted that data do not support a universal recommendation of screening for all women aged 40–49 years and women need to be advised of risks and benefits. A minority report came to the opposite conclusion on the former and agreed with the latter. This was not well received. The director of the National Cancer Institute criticized the majority report and the US Senate voted 98-0 in a non-binding action against it. Fletcher described these events in a colorful comparison to Alice in Wonderland.[15]

The resultant controversy became even more complicated when a later paper raised doubts about the value of mammography screening for women of all ages.[16] Now, in the US, despite conflicting data, screening starts at age 40 or earlier. In most of Europe, it starts at age 50.

It is surprising that during this heated controversy, no attention was paid to the paradoxical breast cancer mortality surge for younger women invited to undergo screening.[11] Meta-analysis of trial data by Cox (shown in Fig. 1) indicates a mortality increase in the screening arms of up to 0.15 deaths per 1000 screened subjects. That begins in the third year (where it is maximum) and extends to the 11th year. While the possibility that random occurrence cannot be excluded, there is a significant excess mortality ratio of screened to unscreened at the 3 year point of 2.4 (1.1–5.4, 95% CI). No other individual years show statistically significant disadvantages as shown in Fig. 2.

Breast cancer is known as a heterogeneous disease. What is causing apparently healthy young women to die from breast cancer three years after the start of screening?

Rather than a controversy, we looked upon this situation as a scientific paradox and research opportunity in that data do not agree with current theories. The scientific method instructs us to re-examine the theory when theory and data disagree.

To help understand this paradox, we studied relapse patterns using a breast cancer database of 1173 pre- and postmenopausal, node-negative and -positive patients treated with surgery only and having 16–20 years of follow-up. This approach is relevant since at least five of the eight screening trials began before the widespread use of adjuvant chemotherapy in approximately 1980.

Methods and patients

All patients who from 1964 through 1980 entered into three different clinical trials at the Milan

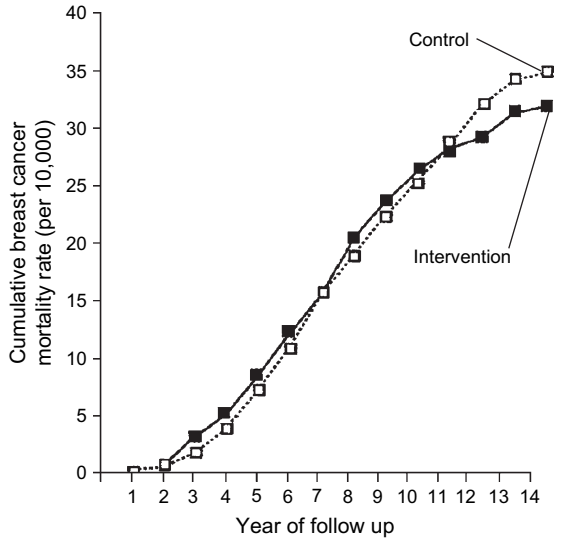

Figure 1 Meta-analysis data for six screening trials for younger women from Cox showing the cumulative breast cancer specific mortality per screened individual and the equivalent mortality per unscreened control. In five of these trials the age at entry was 40—49 years and it was 45—54 year in the other. This figure is based on over 800,000 person-years of experience in each of the screened and control arms. The early disadvantage to screened young women of approximately 0.15 deaths per 1000 screened young women is typical of results seen in all trials. In conjunction with data shown in Fig. 2, the significant disadvantage first appears 3 years into the trial where it is maximum. Modified from Cox.[11]

Cancer Institute, with mastectomy alone as primary treatment for operable breast cancer, were retrospectively evaluated. Before surgery all patients underwent standard staging: complete physical examination, X-ray study of chest, skull, spine, and pelvis, bilateral mammography, ECG, complete

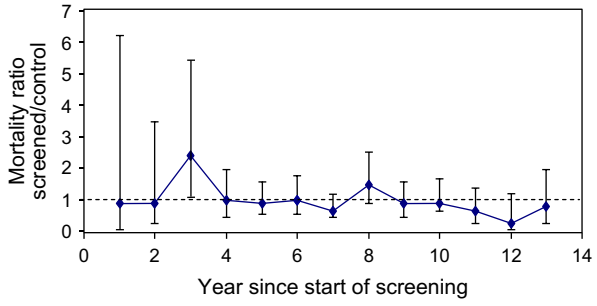

Figure 2 Yearly ratio of mortality in the screened arms to control arms for young women as described in the caption to Fig. 1. There are few events in the first two years accounting for the large error spread. The dashed line at 1.0 represents equal deaths among screened and unscreened controls in any year. The value at 3 years is the only point significantly different from 1.0. Data are from Cox.[11]

Table 1 Distribution within the Milan database of tumor size among the subsets for T1 (<2 cm diameter), T2, and T3 (>5 cm diameter)

	T1	T2	T3	All
Premenopausal	222 (43%)	264 (51%)	30 (6%)	516
Postmenopausal	237 (36%)	364 (55%)	56 (9%)	657
All patients	459 (39%)	628 (54%)	86 (7%)	1173

hemogram and routine biochemical tests. Primary tumor was treated by radical or modified radical mastectomy and no patient received postoperative radiotherapy or chemotherapy.

Menopausal status was defined as ''postmenopausal'' if one year was elapsed since the last menstrual period. The patients were clinical presentation cases, not screening detected. The number of patients included was 1173, and of these, 520 relapsed. Median age at diagnosis was 52 years with a range of 23—82. Distributions are shown in Tables 1 and 2. The representation of patients in the various tumor size and nodal groupings are similar between pre- and postmenopausal subjects.

Results

These data on 1173 untreated early stage breast cancer patients are mature since the follow-up is 16—20 years. Thus it can be assumed that nearly all relapse events have occurred.[17,18]

Surgical cure rates grouped by tumor size and grouped by the number of positive nodes are shown in Table 3. There is no statistical difference between pre- and postmenopausal patients in their long-term prognosis as grouped by tumor size or number of positive nodes. Thus, surgical cure rates were independent of menopausal status.

Relapse data are presented in Fig. 3 as the raw number of distant relapse events grouped in serial bins of 10-month duration. The a posteriori choice to use 10 months as bin size resulted from a comparison of using bins sizes of 6, 10, 14, and 18 months. Small bin sizes show excessive noise while

Table 2 Distribution of nodal status among the subsets

	$N = 0$	$N = 1—3$	$N > 3$	All
Premenopausal	265 (51%)	158 (31%)	93 (18%)	516
Postmenopausal	333 (51%)	184 (28%)	140 (21%)	657
All patients	598 (51%)	342 (29%)	233 (20%)	1173

Table 3 Percentage of patients who eventually relapsed in the mature $N = 1173$ Milan database grouped by tumor size and by the number of positive lymph nodes for pre- and postmenopausal patients

	T1 (%)	T2 (%)	T3 (%)	$N = 0$ (%)	$N = 1-3$ (%)	$N > 3$ (%)
Premenopausal	41	60	70	30	66	84
Postmenopausal	38	56	62	25	66	84

From a difference of proportions hypothesis test, in each case and overall, there is no statistically significant difference between the two menopausal states in cure rates. Thus if a patient had x nodes positive and y tumor size, the long-term relapse probability was independent of menopausal status.

large bin sizes tend to mask structure. Ten-month bins were chosen to optimize the display of structure in the time dependent data.

The frequency of relapse has a double-peaked distribution. There is a sharp peak at 18 months, a nadir at 50 months and a broad peak at 60 months with a long tail extending to 15–20 years. Patients with larger tumors more frequently relapse in the first peak while those with smaller tumors relapse equally in both peaks. Specifically, for T1 tumors (<2 cm diameter) 50% of all relapses are in the first peak, for T2 tumors 75% of relapses are in the first peak, and for T3 tumors (>5 cm diameter) 83% are in the first peak.

When we compared these temporal relapse data between premenopausal patients and postmenopausal patients, the relapse pattern differed markedly but only in the initial period following resection and particularly so for patients with positive axillary lymph node involvement.[19] That is, the temporal relapse pattern had menopausal status dependent features. In premenopausal patients with node-positive disease, 20% relapsed within the first 10 months following resection. That is a far higher percentage than for any other grouping. For comparison, in that first 10-month period, the relapse rate was five times higher for node-positive patients as node-negative patients.

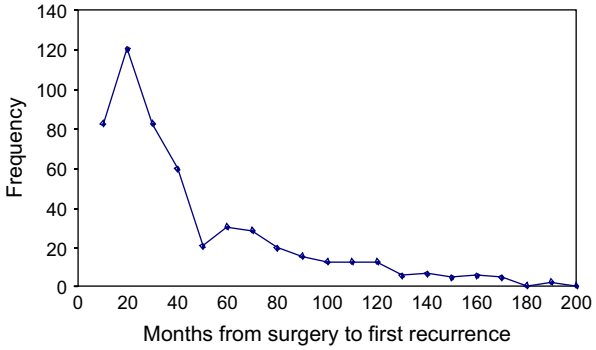

Figure 3 Milan database relapse frequency for distant plus local relapses. Data are grouped in 10-month wide bins.

Also in that same period, the relapse rate was twice as high for premenopausal as postmenopausal patients. So the high frequency of relapse in the first 10 months after surgery was mainly peculiar to premenopausal node-positive patients. See Table 4 for more details.

The Milan data are shown in Fig. 4 in the more usual disease-free-survival format. A subtle flattening at 4 years marks the nadir between the two peaks. That might explain why the bimodal pattern could be so often overlooked. While we have not conducted a thorough literature search, bimodal relapse patterns similar to what is seen in Figs. 3 and 4 have been identified in some (but not all) disease-free survival and hazard of relapse databases for untreated patients.[20–26] A recent study using a San Antonio database that is larger than the Milan database reported that a statistically significant bimodal relapse distribution is identified with similar features.[27] However, using a third database from Villejiuf, another analysis reported no such bimodal pattern.[28] All three databases were tested using different methodologies. From our perspective all these data are not too dissimilar. We have initiated a collaborative project to repeat these studies but with common methodologies.

Predictions from our previously reported computer simulation of the Milan bimodal relapse data are that breast cancer growth often includes periods of temporary dormancy. This is consistent with many reports.[29–38] The second peak is the

Table 4 Percentage of all distant relapses that occur in the first 10 months after surgery in Milan database

	0 nodes positive (%)	1–3 nodes positive (%)	>3 nodes positive (%)
Premenopausal	4	26	28
Postmenopausal	6	12	18

These very early relapses are associated with premenopausal status and positive nodes.

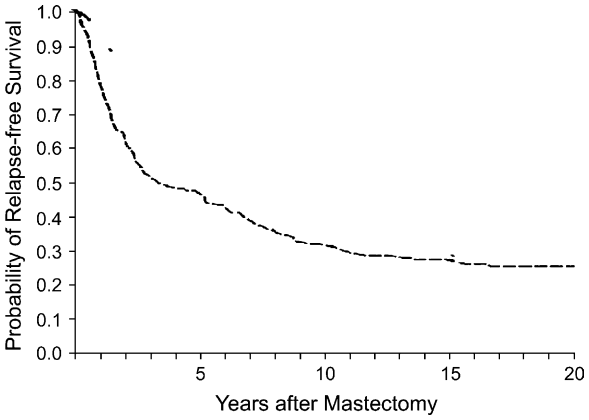

Figure 4 The same data as shown in Fig. 3 but presented in disease-free survival format. The 50-month nadir from Fig. 3 appears as a subtle flattening of disease-free survival before the relapses increase again at the 5-year point. Modified from Bonadonna et al.[59]

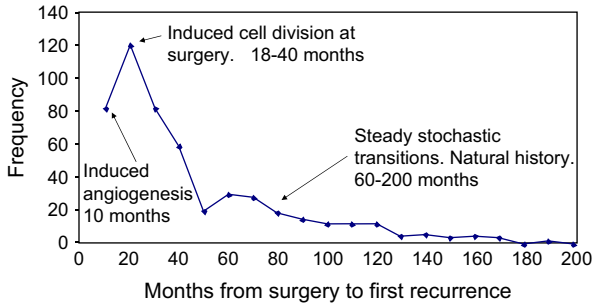

Figure 5 The Milan data from Fig. 3 are shown together with the interpretation resulting from the computer simulation. The main difference between premenopausal and postmenopausal patients is that surgery apparently stimulates angiogenesis of dormant distant disease for a significant fraction of premenopausal and node-positive patients, accelerating disease by a median of two years.

natural history of the disease. These relapses result from steady stochastic transitions from single cells (dormancy half-life of 1 year) progressing to an avascular micrometastasis (dormancy half-life of 2 years) to a growing lesion that eventually becomes detected as a relapse.

The top of the second peak (at 60 months) marks when the benefit of surgery is first seen. That is, the time that it takes a newly seeded malignant cell to become a detectable lesion is so long that the benefit of surgery, that stops the seeding process, does not appear as a reduction in relapses until 5 years have passed in a patient population. This process may be thought of as a metastatic pipeline that is so long that it is fully 5 years after the entrance spigot is turned off before the pipeline is depleted. The first peak is too sharp to be the result of steady stochastic transitions. Some breaking of dormancy had to occur at surgery to explain the first peak. The computer simulation results are shown in Fig. 5 superimposed on the data already shown in Fig. 3.

Two previously unreported surgery-accelerated relapse modes comprise the dominant first peak. This is consistent with some reports for animal models and human cancer.[36–41] In the first 10 months, there are relapses due to avascular micrometastases (preexisting at primary tumor detection) that are stimulated to vascularize at surgery. This mode is prominent only for premenopausal node-positive patients in which case over 20% of patients relapse in this manner. The remainder of events in the first peak are single cells that are dormant at primary detection and are induced to divide as a result of surgery. These then must undergo a stochastic transition to an eventual growing metastasis. This mode is very common — occurring for 50–83% of relapsing patients increasing with tumor size but independent of age.

With this theoretical insight from the computer simulation studies, we turned our attention to the trials of early detection of breast cancer. Mammography screening was first studied in a large randomized controlled trial in New York (the Health Insurance Plan of Greater New York or HIP trial) in the 1960s[3] and was further assessed in other randomized trials (Malmo, Two-County, Stockholm, Goteborg) in Sweden in the 1970s and 1980s.[4–6] The Swedish trials (excluding a Kopparberg segment of the Two-County study) have been recently reviewed by an Overview Committee that confirmed fundamentally the results previously reported by the individual research groups.[42] Even the results of a UK trial (Edinburgh) were quite similar although this trial has been criticized for a randomization bias.[7,43] Trial results for the New York, Swedish overview and Edinburgh trials are shown in Figs. 6–8.

As already stated, computer simulation suggests that the removal of a primary breast tumor from premenopausal node-positive women triggers the growth of temporarily dormant distant micrometastases in approximately 20% of cases. Since the yield is relatively high at the initial screen in a previously unscreened population, such relapses would appear prominently in a screening trial within 1 year after the start of screening. However, we need to translate these relapse events into mortality events in order to compare to published data from all screening trials. Using published screening yield rates and knowing that survival after relapse is approximately 2 years, we have calculated that this putative surgery-induced growth could explain an additional 0.11 deaths

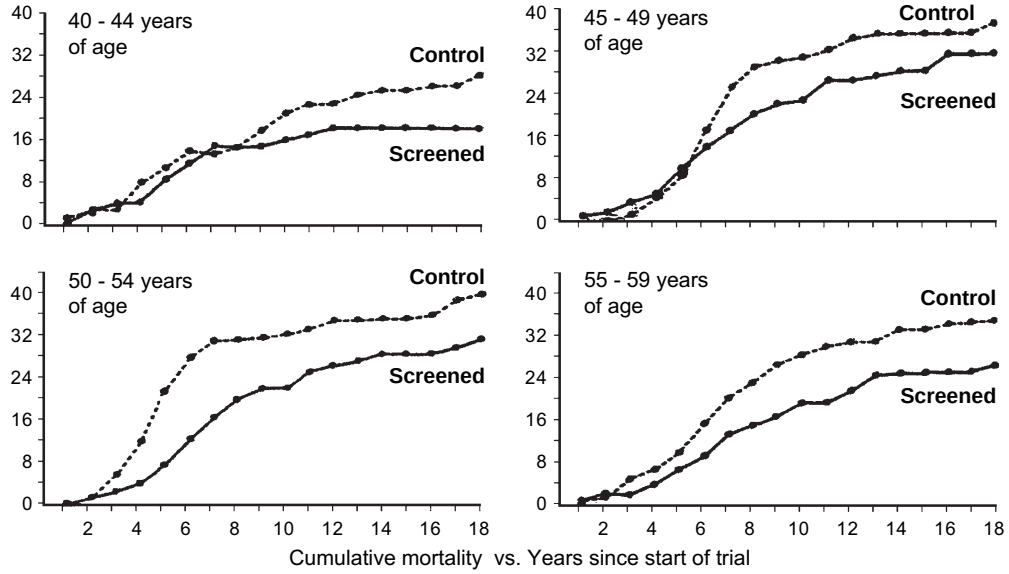

Figure 6 The Health Insurance Plan (HIP) of Greater New York was the first randomized clinical trial of mammography. These cumulative mortality data are modified from Shapiro.[3] The early appearing advantage of mammography for women aged 50—59 is seen together with the delayed advantage for women aged 40—49. A two-year shift to the right in the mortality curve for women aged 40—49 would provide early detection advantage very similar to the 20—30% advantage seen for women aged 50—59.

per 1000 screened women aged 40—49 that occurs in the third year after the start of screening.[19] This is approximately what is observed in trials as can be seen in Figs. 1,7, and 8. The HIP data (Fig. 6) are not published in a convenient format for this comparison, but the excess mortality is quantitatively the same as the other trials seen in Figs. 7 and 8.

While that excess mortality magnitude may seem small, it is comparable to the US age adjusted death rate from breast cancer of 0.24 per 1000 women.

Figure 7 Five of the mammography trials were conducted in Sweden. These data are the combined results of these trials and constitute the bulk of the mammography data. Data for women aged 40—49 are shown. The early excess mortality for the screened population is apparent beginning in the third year and continuing until the seventh year when a clear advantage begins to appear. In the third year, the apparent disadvantage of screening is approximately 0.1 per 1000 screened women aged 40—49, in agreement with calculations. Modified from Larsson et al.[5] As in Fig. 6, a two-year shift to the right would produce 20—30% mortality advantage for women aged 40—49.

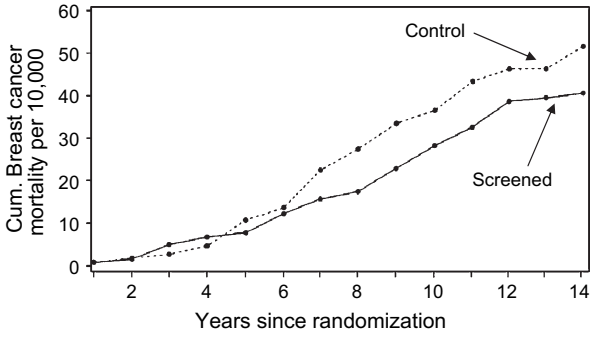

Figure 8 The Edinburgh clinical trial of mammography is shown. This trial has been criticized for a randomization bias. However, it still shows the same pattern as in the HIP (New York) trial in Fig. 6 and the Swedish overview in Fig. 7. The disadvantage to the intervention group is maximum in the third year and is approximately 0.1 per 1000 screened age 40—49 women. Modified from Alexander.[7] As in Figs. 6 and 7, a two-year shift to the right would produce 20—30% mortality advantage for women aged 40—49.

As an additional opportunity to compare the computer simulation with the trial data, we note that a two-year shift to the right of the age 40–49 screened population in Figs. 1, 6–8 would result in mortality advantage to screening similar to what is found in trials for women age 50–59. This is consistent with the previously mentioned two-year acceleration in disease due to termination of dormancy in avascular micrometastases.

We proposed that the biological mechanism of the surgical influence on the metastatic development could be a surge of angiogenesis resulting from the removal of inhibitors, the appearance of growth factors or other such effect. This would synchronize some patients to the time when screening begins – which might explain a subset with homogeneous behavior in a heterogeneous disease as seen in Fig. 2. This mechanism is proposed as an explanation of the paradoxical mammography data for women aged 40–49 and is consistent with the bimodal relapse pattern observed.

Conclusions

We have discussed a bimodal relapse pattern for untreated breast cancer patients and the mammography paradox for women age 40–49. Analysis of these data provides indirect evidence that surgery to remove a primary breast tumor can induce angiogenesis of dormant distant disease. Testing the hypotheses presented here should be a high priority. If they prove to be correct, various approaches could be taken to provide the full benefit of screening to women age 40–49.

Clinical trials could be designed to test whether premenopausal women given an antiangiogenic drug during the critical few days before and after surgery fared better. In addition, surgery-induced angiogenesis in breast cancer is very likely regulated by hormones since it occurs much more frequently in premenopausal patients than in postmenopausal patients. This strongly suggests that hormone related interventions, of which there are several possibilities, might prove very useful.[44–48] If there is concern that an antiangiogenic treatment after surgery could interfere with wound healing, a hormone-based method could be a good option.

An interesting off-topic speculation resulting from this study is a possible evolutionary based explanation of why there is dormancy of distant micrometastases in premenopausal women with primary breast tumors. Before the historical advent of surgical intervention in breast cancer,[49] this effect would allow a female of childbearing age with a primary breast cancer and this trait to live an extra two years and thus have more offspring than if she did not have that trait.

Another off-topic subject is that our conclusions might provide a scientific basis for the often-debunked myth that ''cancer spreads when the air hits it''.[50] The effect we describe would make it seem as though cancer spreads after surgery, while of course the cancer had already spread but only escapes long-lasting pre-angiogenic dormancy as a biological sequel of surgery.

Our results suggest that the biology of early detection is more complex than originally thought.[51,52] Early detection sometimes produces disappointing results as seen in large clinical trials[9] and community-based screening.[53]

The screened population is far from homogenous with regard to risk and benefit of early detection. In light of our findings, we suggest that until this is better understood and resolved, guidelines for early detection of breast cancer for young women be reconsidered. At the very least, women need to be advised of this information as part of an informed consent to mammography.[54] Well-intentioned sweeping this problem under the rug[15,55,56] has not been helpful.

More research is needed to confirm our findings. If true, in addition to the impact on early detection, a comprehensive treatment plan for breast cancer would probably need to take into consideration the possibility that surgery could stimulate tumor growth including inducing angiogenesis.[57,58]

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